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Award Number: DAMD17-99-1-9110

TITLE: Src-JNK Potentiation of Estrogen Receptor AF-1; Mechanism, and Role in Estrogen Action in Breast Cancer

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REPORT DATE: August 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2000	Annual (1 Aug 99 - 31 Jul 00)

4. TITLE AND SUBTITLE
Src-JNK Potentiation of Estrogen Receptor AF-1; Mechanism, and Role in Estrogen Action in Breast Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9110

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The activity of Src tyrosine kinase is commonly elevated in breast cancer and breast cancer cell lines, but the significance of this elevation is not known. In preliminary studies we found that increasing Src activity potentiates the ability of the estrogen receptor to stimulate transcription of target genes, and thereby alter cellular functions, and that Src must activate the JNK proteins in order to potentiate the estrogen receptor. We have two objectives. One is to understand in detail the molecular pathway whereby activated Src and JNK leads to an increase in estrogen receptor activity. A second objective is to understand the potential role of Src in estrogen induced mammary ductal development and estrogen-induced breast cancer proliferation.

In the first year of this study we have largely accomplished the first of these objectives. We found that Src activated the first 100 amino acids in the estrogen receptor AF-1 function as its main target, that to do so Src worked primarily through the JNK family of MAP kinases, and that the JNK target appears to be the CBP/p160 coactivators coactivators that mediate AF-1 action, and not the estrogen receptor itself. These results suggest potential new targets for anticancer therapy.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer/Src tyrosine kinase/estrogen receptor/JNK/AF-1	67
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Introduction, Src year 1 annual report

The activity of Src is commonly elevated in breast cancer and breast cancer cell lines, but the significance of this elevation is not known. Many breast cancers and breast cancer cell lines also contain estrogen receptors and are stimulated to grow by estrogen. In preliminary studies we found that increasing Src activity potentiates the ability of the estrogen receptor to stimulate transcription of target genes. These prelimary studies suggest a possible connection between Src activity and estrogen receptor activity that we propose to explore. We have two objectives. One is to understand in detail the molecular pathway whereby activated Src and JNK leads to an increase in estrogen receptor activity. A second objective is to understand the potential role of Src in estrogen induced mammary ductal development and estrogen-induced breast cancer proliferation.

(5) BODY

We had thee tasks:

Task 1. To identify the pathway leading from Src activated JNK to increased estrogen receptor AF-1 activity (months 1-18).

Task 2. To determine the role of Src in estrogen induced mammary gland ductal epithelial proliferation and branching at puberty. (months 24-36)

Task 3. Determine the role of Src in estrogen stimulated tumor growth by studying the effects of estrogen on the proliferation of breast cancer cell lines in which Src has been made nonfunctional (months 24-36)

In this first year of the project most of our progress has been on task 1. We have accomplished much of this task. Our progress is detailed in the appended manuscript, Potentiation of estrogen receptor AF-1 by Src/JNK through a serine 118-independent pathay, which is now in the final stages of review for Molecular Endocrinology [1]. In particular, we have carefully confirmed and documented that the ER AF-1 function is the Src/JNK target. We have demonstrated that the target maps to a subdomain of AF-1, amino acids 1-100. We also demonstrate that the Src/JNK target does not involve phosphorylation of S118, the target of ERKs. Finally we show that the CBP/p160 coactivator complex appears to be the JNK target (ref) rathar than ER itself.

We also desired to map potential sites of phosphorylation in CBP and p160s by JNK as part of task 1. Unfortunately this is proving more difficult than anticipated because of multiple sites of phosphorylation. We will report more fully on this aspect of the task in a future report.

We have not as yet worked on task 2.

Task 3 was not anticipated to begin in year one, but we have made some unexpected progress towards this aim, which is also described in the appended manuscript by Feng et al. We found that in MCF-7 breast cancer cells that the activity of the endogenous ER was enhanced by elevating Src activity by transfection with v-Src. This establishes that in breast cancer cell lines that ER activity is under Src regulation, and provides a critical control for the experiment in which we determine whether reducing Src activity in these cells reduces ER action on transcription and proliferation.

(6) KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that the Src tyrosine kinase, known to be elevated in breast cancer, increase the activity of the estrogen receptor, a critical proliferative factor in many breast cancers.

- Demonstrated that the pathway from Src leads to JNK, a MAP kinase, and then to estrogen receptor activator function 1, most likely through the coactivators CBP/p160.

- Demonstrated that increased Src activity potentiates estrogen action in human breast cancer cells in culture.

(7) REPORTABLE OUTCOMES.

One manuscript, by Feng et al, " Potentiation of estrogen receptor AF-1 by Src/JNK through a serine 118-independent pathway, "which is currently in the final stages of review for Molecular Endocrinology and is appended.

(8) CONCLUSIONS;

Our main conclusions are first, that the Src tyrosine kinase, known to be elevated in breast cancer, increases the activity of the estrogen receptor, a critical proliferative factor in many breast cancers. This activity of Src is seen in transfected cells of various kinds and also seen in MCF-7 cells expressing their own estrogen receptor. This conclusion suggests that overactivity of Src in breast cancer may be leading to hyper activity of the estrogen receptor and may be playing a key role in estrogen dependent breast cancer. "So what?" If the conclusion is correct it will suggest new ways to intervene in breast cancer prevention and therapy.

We also conclude that the pathway from Src leads to JNK, a MAP kinase, and then to estrogen receptor activator function 1, most likely through the coactivators CBP/p160. "So what?" These conclusions suggest that drugs that might target any of the steps in this pathway, might be useful as potential treatments for hormone dependent breast cancer.

(9) REFERENCES

1. Feng, W.J. *et al.* Potentiation of estrogen receptor AF-1 by Src/JNK through a serine 118-independent pathway. (submitted).

Potentiation of Estrogen Receptor AF-1 by Src/JNK Through a Serine 118-independent Pathway.

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Abstract:

Estrogen receptor (ER) may be activated either by ligand or by signals from tyrosine kinase-linked cell surface receptors. We investigated whether the non-receptor Src tyrosine kinase could affect ER activity. Expression of constitutively active Src or stimulation of the endogenous Src/JNK pathway enhances transcriptional activation by the estrogen-ER complex and strongly stimulates the otherwise weak activation by the tamoxifen-ER complex. Src affects ER activation function 1 (AF-1), and not ER AF-2, and does so through its tyrosine kinase activity. This effect of Src is mediated partly through a Raf/MEK/ERK signaling cascade and partly through a MEKK/JNKK/JNK cascade. Although, as previously shown, Src action through activated ERK stimulates AF-1 by phosphorylation at S118, Src action through activated JNK neither leads to phosphorylation of S118, nor requires S118 for its action. We therefore suggest that the Src/JNK pathway enhances AF-1 activity by modification of ER AF-1 associated proteins. Src potentiates activation functions in CBP and GRIP1, and we discuss the possibility that the Src/JNK pathway may enhance the activity of these coactivators, which are known to mediate AF-1 action.

Introduction

The action of estrogen receptor alpha (hereafter, ER) is regulated both by binding of ligand and by inputs from signal transduction cascades. Binding of estrogen to ER frees it from a complex with heat shock proteins, and allows ER to bind estrogen response elements (EREs) in the promoter region of target genes (for review see [1]). ER then stimulates transcription via the concerted action of the AF-1 activation function in its amino terminus and the hormone-dependent activation function, AF-2, that lies within the LBD. The antiestrogen tamoxifen allows release from heat shock proteins and ERE binding, but blocks AF-2 (reviewed in [1,2]). Tamoxifen allows weak AF-1 activity, but in many cases this is insufficient to increase gene expression [3]. Other antiestrogens, such as raloxifene and ICI 182,780 (ICI), allow neither AF-1 nor AF-2 activity [4,5]. Both activation functions work by recruiting a coactivator complex to the promoter (reviewed in [1,6,7]). The complex contains a p160 protein, such as SRC-1(N-CoA1) [8,9], GRIP1 (TIF2, N-CoA2) [10-12], or p/CIP (AIB1, ACTR, TRAM-1, RAC3) [13-17], p300/CBP [9,18-20] and p/CAF [21-23] (for review see [7]). AF-1 binds the C-terminus of the p160 component [24], whereas estrogen bound AF-2 binds tightly to a separate regions of the p160s (the NR boxes) [13,25-29]. The coactivator complex, once recruited, is presumed to stimulate transcription by interactions with components of the basal transcription machinery and also by its histone acetyl-transferase activity that may remodel chromatin and allow access to the transcriptional template [15,30-32].

ER activity is also stimulated by signaling pathways that are activated when growth factors, such as EGF and IGF1, bind their tyrosine kinase linked receptors. Growth factors are sometimes sufficient to activate ER in the apparent absence of ligand [33-36]. More commonly, growth factors synergize with ligand by enhancing AF-1 activity. EGF binding to the EGF receptor results in sequential activation of Ras, Raf, MEK, and the MAP kinases ERK1 and ERK2, which phosphorylate ER at Serine 118 in the N-terminal domain and potentiate AF-1 activity [36-38]. Mutation of S118 to alanine blocks ER phosphorylation by MAP kinases and potentiation of AF-1 action by growth factors [36-38]. Furthermore, the S118A mutation also reduces basal

phosphorylation of S118 by unspecified kinases [38] and decreases basal AF-1 activity [39,40]. Phosphorylated ER AF-1 shows enhanced binding to the p68 RNA helicase, which is thought to account for its enhanced transcriptional activity [41]. AF-1/GRIP1 interactions map to the N-terminal region of the ER AB domain, which has not been implicated in growth factor enhancement of AF-1 activity, and are unaffected by mutations in serine 118 [24].

ER is likely to be subject to signal transduction inputs during developmental and repair processes and may also be subject to abnormal stimulation by such pathways during pathological states, such as cancer. Many breast tumors exhibit elevated expression of growth factors such as EGF, Her2/neu, IGFs and their receptors [42-45]. Furthermore, more than 80% of primary breast cancers show increased activity of the non-receptor Src tyrosine kinase activity compared with normal breast tissue [46-49], reviewed in ([50]). Elevated Src activity leads to activation of multiple signal transduction cascades (reviewed in [51-53]). Src activates both the ERK and JNK subgroups of MAP kinases (for examples see [54,55]). Src activates ERKs presumably via Ras and the Raf/MEK/ERK kinase cascade (see, for example, [54]). It also activates JNKs, presumably via Rac-1 and related GTPases, and the sequential activation of the MEKK/JNKK/JNK kinase cascade [56]. In light of these reports we investigated whether activated Src enhances ER action. We find that Src does so, and that it specifically enhances ER AF-1 activity via two independent mechanisms. One involves phosphorylation at S118 via Src activation of the Raf1-MEK-ERK pathway. The other is mediated by Src activation of the MEKK-JNKK-JNK pathway, the target of which does not appear to be S118, or even ER. We discuss the possibility that JNKs target one of the several coactivators that associate with the ER.

Results

Src potentiates ER AF-1.

Src activity in breast cancer cells and cell lines is often elevated up to 30-fold [49]. To examine the effects of elevated Src activity on ER action, we transfected expression vectors for v-Src and

control vectors into HeLa cells along with expression vectors for ER and an ERE responsive reporter gene (ERE:HSV-TK-CAT). v-Src is a viral derivative of cellular Src (c-Src) which has at least 10-fold higher kinase activity than c-Src. Fig. 1A shows that v-Src potentiated ER transcriptional activity by two fold in the presence of 17ß-estradiol (E2), a primary estrogen agonist activating both AF-1 and AF-2. More strikingly, v-Src also enhanced ER transcriptional activity by about fifteen fold in the presence of tamoxifen (Tam), an ER ligand that inhibits AF-2, but allows AF-1 activity [3]. v-Src also enhanced ER transcriptional activity in the presence of raloxifene (Ral), an ER ligand that allows partial AF-1 activity, but did not enhance ER transcriptional activity in the presence of ICI 182,780, an ER ligand that blocks the activities of both AF-1 and AF-2 [3,57-59], (Fig. 1A, inset with expanded scale). Over the course of this study, v-Src enhanced the overall levels of estrogen response by between 50% and four fold, but consistently yielded larger enhancements of tamoxifen response. Consequently, v-Src increased the overall level of tamoxifen response from a tiny percentage of estrogen response to 15-40% of the overall estrogen response.

To ask whether v-Src effects upon ER activity could be explained by increases in ER levels, we performed western blots on extracts of cells that had been transfected with different amounts of ER in the presence and absence of transfected v-Src. Fig. 1B shows that the amount of ER increased as a function of transfected ER expression vector in the absence of v-Src, and that v-Src increased ER levels by about three fold. In parallel, v-Src gave much stronger potentiation of ER action at the ERE responsive reporter gene, even at ER levels that were optimal for estrogen and tamoxifen response (Fig. 1B lower panel). In particular, the ER activity obtained in the presence of 3µg of transfected ER and v-Src exceeded the ER activity obtained in the presence of 10µg of transfected ER and no v-Src, even though the former contained lower amounts of ER protein. Thus, v-Src increases ER transcriptional activity, especially in the presence of tamoxifen.

To ask whether v-Src enhancement of ER activity might occur under more physiological conditions, we first examined the amount of v-Src required for enhancement of ER activity. v-Src potentiation of tamoxifen response could be observed with as little as 300-600ng of transfected v-

Src expression vector (Fig. 1C). We then examined v-Src action upon the ERE responsive reporter in MCF-7 breast tumor cells, which express endogenous ER (Fig. 1D). Here, endogenous ER showed significant constitutive activity, which was further elevated by addition of estradiol but completely suppressed by tamoxifen and ICI. In the presence of transfected v-Src, both the constitutive and estrogen-dependent transcriptional activity were modestly elevated and, once again, tamoxifen-dependent transcriptional enhancement was strongly increased. Thus, v-Src enhances the activity of the estrogen-ER and tamoxifen-ER complexes in breast cells, just as it does in HeLa cells.

That the ER response to v-Src overexpression is more dramatic in the presence of tamoxifen or raloxifene than in the presence of estrogen, and that there is no response in the presence of ICI suggests that ER AF-1 might be a primary action target of v-Src. To further investigate whether the target of v-Src action was AF-1 or AF-2, we examined the effect of v-Src overexpression upon the activity of a reporter gene with a promoter containing a TATA box and multiple binding sites for the yeast GAL4 protein (5xGALRE-E1b-tata-LUC). This reporter was then activated with the DNA binding domain of GAL4 fused to the ER A/B region containing AF-1 (GAL4-ER(A/B)), to the ER LBD containing AF-2 (GAL4-ER(LBD)), or to VP16 (GAL4-VP16). As shown in Fig. 1E, v-Src potentiated the GAL4-ER(A/B) transcriptional function around 30-40 fold, but had only a minimal effect on GAL4-LBD or GAL4-VP16. Like v-Src action upon the tamoxifen-liganded ER, v-Src potentiation of AF-1 activity could be detected at optimal levels of transfected GAL4-ER(A/B) expression vector (data not shown). Thus, v-Src strongly enhances ER AF-1 activity. These observations are consistent with the strong activation of the tamoxifen-bound ER by v-Src and confirm that the A/B domain of ER, which contains AF-1, is the v-Src target.

Src action Upon ER is Independent of Tyrosine 537.

Some reports have suggested that Src may be able to directly phosphorylate ER at tyrosine 537 (Y537) within the ER-LBD [60-62], although this remains controversial. In fact, mutations within

Y537 were later shown to enhance ER activity in the absence of hormone, by allowing ligand-independent interactions of ER AF-2 with its target coactivators [63-65]. To ask whether Y537 phosphorylation was required for v-Src action upon the ER, we examined the effect of v-Src overexpression upon ERs bearing mutations within this residue. Fig. 2 shows that both ER and ER-G400V, an ER mutant that lacks constitutive activity, but is otherwise normal [66], elicited strong estrogen responses and weak tamoxifen responses from the ERE responsive reporter. In parallel, ERs bearing either an arginine (Y537R) or serine (Y537S) substitution at position 537 showed the expected increase in constitutive activity, but retained very little activity in the presence of tamoxifen. In the presence of transfected v-Src, both ER and each of the ER mutants (G400V, Y537R and Y537S) showed modestly enhanced transcriptional activity in the presence and absence of estradiol and a larger enhancement of transcriptional activity in the presence of tamoxifen. We therefore conclude that the weak v-Src enhancement of the activity of the estrogen-ER complex, and the strong enhancement of activity of the tamoxifen-ER complex, are both independent of ER phosphorylation at tyrosine 537.

Src kinase activity is required for potentiation of ER AF-1.

To investigate whether elevated Src kinase activity itself, and not some other feature of v-Src, leads to potentiation of ER action, we examined the effect of various Src expression vectors and tyrosine kinase inhibitors upon ER AF-1 activity. Wild type c-Src and constitutively activated c-Src(Y527F) potentiated the activity of the GAL-ER(A/B) fusion protein by 2~3- and 5-fold, respectively (Fig 3). The kinase-inactive mutant Src(Y295K, Y527F), in contrast, was unable to enhance AF-1. Moreover, enhancement of ER AF-1 by Src was blocked, and even reduced below basal levels, by genistein and Herbymicin A, two inhibitors of Src tyrosine kinase activity (Fig. 3). Control experiments indicated that both of these inhibitors were without effect on GAL-VP16 (data not shown). These results indicate that the intrinsic tyrosine kinase, and not another feature of the v-Src molecule, mediates the enhancement of ER activities. That the kinase inhibitors reduce AF-1

level below basal may further suggest that endogenous Src tyrosine kinase activity might underlie basal AF-1 activity.

Src potentiates AF-1 by both the Ras-Raf1-MEK-ERK and the Rac-MEKK-JNKK-JNK pathways.

Src tyrosine kinase might affect AF-1 either directly, or indirectly through the numerous signal transduction cascades that Src is known to initiate. While Src is generally associated with the plasma membrane, and ER is generally nuclear, these locations are not rigid. In particular, there are reports of ER associated with the cell surface [67,68]. Thus, there is nothing *a priori* to exclude the possibility that Src might directly phosphorylate the ER AF-1 domain. We therefore tested whether a GST-ER(A/B) fusion protein was a substrate for activated c-Src kinase. GST-ER(A/B) was efficiently phosphorylated by activated ERK2 MAP kinase, consistent with other studies [37], but not by activated c-Src kinase (Fig. 4A). Thus, ER is not a direct substrate of Src. We infer that Src must activate ER AF-1 indirectly through phosphorylation cascades.

Because Src is known to cause to MAP kinase activation, and the well known pathway of Ras, Raf-1, MEK and ERK activation enhances AF-1 activity through serine 118 phosphorylation, we asked whether v-Src might enhance ER activity by initiating the Raf-1/MEK/ERK cascade. We first examined the effect of various inhibitors of this pathway upon EGF stimulation of the transcription factor ELK-1, which responds even more dramatically to EGF than does the ER. As shown in Fig. 4B, left, each of these reagents nearly abolished the EGF activation of a GAL-Elk1 fusion protein. PD 98059, a specific inhibitor of MEK activation [69], also blocked EGF activation of GAL-Elk1. Thus, these inhibitors work effectively to block transcription mediated by the ERK pathway in these transfected cells. In contrast, only modest (20-40%, shown) decreases in v-Src effects on GAL-ER (A/B) were detected. Even high levels of transiently transfected expression vectors for each dominant negative protein produced no more than 30-40% inhibition of Src action (data not shown). Similar modest inhibition was obtained with PD 98059. These

results indicate that Src potentiation of ER AF-1 is only partly mediated by the Ras/Raf/MEK/ERK pathway and suggest that another pathway must also be involved.

Because the Rac-MEKK-JNKK-JNK pathway is also activated by v-Src, we then asked whether this pathway might mediate v-Src effects upon the ER. Dominant negative Rac(S17N) or MEKK1(K432M), strongly inhibited v-Src activation of ER AF-1 (Fig. 4C). In a separate experiment a vector for dominant negative JNKK4(K116R) eliminated the induction mediated by short term exposure to v-Src expression or stimulation of the endogenous Src/JNK pathway treatment with the cytokine TNFα (Fig. 4D). These observations indicate that the route from Src to AF-1 proceeds through both the Ras-Raf-MEK and the Rac-MEKK-JNKK pathways and that the ERK and JNK kinases are the true effectors of Src action.

The role of the ERK and JNK kinases was then confirmed by examining the effect of elevated expression of these kinases on Src action. Overexpressed JNK1 and ERK2 synergized with v-Src to activate ER AF-1 (Fig. 4E). Neither JNK nor ERK alone was sufficient to enhance AF-1 activity, suggesting that both required inputs from upstream kinase cascades to enhance AF-1 activity. In contrast, overexpression of another MAP kinase, p38, which is also activated by JNKK1 [70], inhibited, rather than synergized with, the v-Src activation of ER AF-1. While the mechanism of this dominant negative effect of p38 is unknown, the fact that p38 acts as a dominant negative indicates that p38 does not mediate Src activation of AF-1. In summary, these studies indicate that the route from Src to AF-1 proceeds through both a Ras-Raf-MEK-ERK and a Rac-MEKK-JNKK-JNK pathway.

Src potentiates AF-1 both by ERK mediated phosphorylation of S118 and by a JNK mediated, S118 independent, pathway.

As noted previously, activation of the Ras-Raf-MEK-ERK pathway leads to phosphorylation of ER S118 by ERKs. We therefore investigated whether S118, or the nearby S104 and S106, played similar roles in Src activation of AF-1. We first examined the effect of S118 mutations to glutamic acid (E), which mimics the negative charge of phosphorylated serine and allows stronger

ER AF-1 binding to p68 RNA helicase [36, 41], or arginine (R), which blocks S118 phosphorylation. As expected, ERS118E showed a slight increase in tamoxifen response relative to ER, and ERS118R showed no tamoxifen response (Fig. 5A). In parallel, transfected v-Src gave a much stronger enhancement of tamoxifen response, suggesting that the increased negative charge of the ER-S118E mutant was insufficient to the mimic the enhancement of AF-1 activity that is obtained with v-Src. Furthermore, ERS118E dependent tamoxifen responses were still strongly enhanced by v-Src and ERS118R only reduced the overall level of tamoxifen response in the presence of v-Src by about 50%. These results suggest that v-Src potentiation of AF-1 activity is partially insensitive to mutation of S118. Mutation of S118 to alanine also decreased the ability of v-Src to potentiate GAL-ER (A/B) by 20 to 40% (Fig. 5B and data not shown). There was no further decrease in AF-1 activity when all three serines were mutated to alanine (S104,106,118A). Thus, v-Src enhancement of isolated AF-1 is also partially insensitive to mutation of the AF-1 phosphorylation sites.

It is striking that Src has a major effect on AF-1, even in the presence of the triple serine mutation. To test whether this effect is mediated by the non-ERK (that is, JNK) part of the Src-AF-1 pathway, we examined Src activation of wild type and triple mutant GAL-ER (A/B) in the presence of PD 98059. This inhibitor reduced Src potentiation of AF-1 activity by about 50%, as did the mutation of the three serines (Fig. 5C). However, PD 98059 had no effect on the residual Src activation of the triple serine mutant. This result indicates that the portion of the Src effect that is independent of S118 phosphorylation is also ERK independent. Because JNK activation is not blocked by PD 98059 [69], and data not shown), we infer that the serine-independent portion of AF-1 activation is likely to be the portion that is mediated by JNKs.

To examine the role of JNKs more directly, we examined the ability of Src and overexpressed JNK kinase to cooperate in AF-1 activation. As shown in Fig 5D, v-Src and JNK cooperated to enhance the activity of GAL-A/B S118A mutant even more strongly than v-Src alone. Thus, the Src-JNK pathway enhances AF-1 activity in a manner that is independent of phosphorylation at S118. Surprisingly, ERK1 now specifically inhibited v-Src action at the GAL-

A/B S118A mutant, much as p38 inhibited v-Src action upon wild type AF-1 (Fig. 4E). Again, we do not have a ready explanation for this effect, but speculate that transfected ERKs could inhibit other second messenger inputs to AF-1, and that this effect is only detectable when their stimulatory effect upon ER AF-1 activity is abolished. Nonetheless, taken together, our results suggest that v-Src enhances AF-1 activity via two independent pathways, one that involves ERKs and targets the AF-1 serine cluster and one that involves JNKs and is independent of the serine cluster.

Src activates two separate subdomains of AF-1 which show differential sensitivity to inhibitors of MAP kinases.

AF-1 is complex, and it has been suggested that AF-1 is comprised of independent subdomains [39,57,71]. We therefore tested some of these candidate subdomains for response to Src. We transfected expression vectors for ERs with specific deletions of the N-terminal (AB) domain into HeLa cells and asked whether they would elicit a tamoxifen response from the ERE-CAT reporter gene in the presence or absence of cotransfected v-Src (Fig. 6A). As expected, v-Src enhanced the tamoxifen response obtained with wild type ER by about eight fold, and showed no effect upon an ER truncation that lacked the A/B domain (ΔAB, 185). Transfected v-Src also enhanced the tamoxifen response obtained with an ER truncation that contained amino acids 1-129 by seven fold. This truncation contains all of the ER sequences that are required for AF-1 activity in HeLa cells [57], so this result underscores the notion that v-Src acts upon AF-1. Transfected v-Src enhanced the tamoxifen responses obtained with ERs bearing either the N-terminal (amino acids 1-94) or C-terminal (amino acids 101-185) AF-1 subdomains by about five fold. Furthermore, Src responsiveness of the C-terminal AF-1 subdomain was lost when the region from 101 to 117, which contains the serine cluster, was deleted (117). Together, these observations indicate that v-Src targets two separate sub-domains in AF-1. One lies between amino acids 1-94, and a second lies between amino acids 101-129, which overlaps the serine cluster. Although the role of the serine cluster as a target of second messengers is well established, this is the first direct indication

that the N-terminal region of AF-1 (amino acids 1-94) contributes to second messenger stimulation of AF-1 activity.

Control transfections revealed that PD 98059 completely failed to inhibit the activity of the ER truncation that only contained the N-terminal AF-1 subdomain (amino acids 1-94, Fig. 6B). Thus, v-Src action upon the N-terminal AF-1 subdomain does not proceed through MAP kinase activation, which implies that v-Src action upon this region proceeds through JNK kinases.

Activated JNKs do not phosphorylate the AF-1 domain suggesting an indirect mode of action.

The above studies indicate that Src activates AF-1 through at least two pathways, one of which is mediated through JNK and does not require S118. To investigate whether JNK might phosphorylate another site within AF-1, we tested the ability of JNKs extracted from cells that had been treated with the pro inflammatory cytokine and activator of Src/JNK, TNFα, [70] for their ability to phosphorylate the AF-1 domain *in vitro*. Activated JNKs were able to efficiently phosphorylate a c-Jun substrate *in vitro* (Fig. 7 top), but were unable to phosphorylate an AF-1 substrate, even though this substrate had been efficiently phosphorylated by activated ERK as shown earlier (Fig. 4A). We conclude that activated JNKs do not directly phosphorylate the ER AF-1 domain.

Src can potentiate AF-1 in conditions where AF-1 is mediated by the GRIP1/CBP complex.

Our results show that Src stimulates ER AF-1 activity through JNK kinases, and that the JNKs do not phosphorylate the AF-1 domain. This suggests that the action of the JNK pathway on AF-1 may be mediated by phosphorylation of another protein that mediates ER activity. Basal AF-1 activity is mediated by a complex of a p160 such as GRIP1, along with p300/CBP [24]. The essential contact for this action is between the N-terminal sub-domain of AF-1 and the C terminal

domain of GRIP1. We therefore asked whether v-Src enhanced AF-1 under conditions in which AF-1 is itself enhanced by overexpression of GRIP1 and CBP.

We first cotransfected expression vectors for GRIP1 or CBP along with vectors for GAL-AF-1 and v-Src, and asked whether the coactivators would potentiate v-Src action on the GALRE:luc reporter gene. Overexpression of GRIP1 further enhanced v-Src potentiation of ER AF-1 (7-9 fold, Fig. 8A left). CBP also slightly increased Src effects on AF-1, similar to its action on basal AF-1 [24]. Thus, v-Src synergizes with overexpressed GRIP1 and CBP, suggesting that Src activation of AF-1 can occur when AF-1 activity is mediated by contacts with GRIP1 and CBP. We further examined whether the S118 independent and PD 98059 resistant component of Src action also synergizes with GRIP1. GRIP1 synergized with v-Src to activate GAL-AF-1 with the triple serine mutation S104, 106, 118 A (Fig. 8A, right) both with and without PD 98059. We conclude that the S118-independent and PD resistant component of the Src pathway of AF-1 potentiation is active when AF-1 is mediated by the p160 coactivator GRIP1.

As noted above, both AF-1 and AF-2 both recruit the p160-CBP complex, but the two ER domains contact different surfaces on the coactivators. AF-1 contacts the C-terminal domain of GRIP1 [24], whereas AF-2 contacts one of the multiple p160 NR Boxes, which are located in the middle portion of GRIP1 [13,26-29]. Thus, deletion of the C-terminus of GRIP1 prevents AF-1 activity without affecting AF-2. To test whether AF-1/GRIP1 contacts were needed for v-Src enhancement of AF-1, we overexpressed a C-terminal deletion of GRIP1 (1-1121aa), which lacks the site for AF-1 binding [24]. The GRIP1 mutant missing the C-terminus failed to cooperate with v-Src to enhance AF-1 and reduced activation by v-Src (Fig.8B). Similar observations were also obtained in the presence of tamoxifen activated ER (data not shown). Thus, the C-terminal deleted GRIP1 has a dominant negative effect on Src activation of AF-1. Taken together, these observations confirm that Src can potentiate AF-1 under conditions where AF-1 activity is mediated by contacts with the GRIP1 C-terminus. It may also indicate that Src activation of AF-1, like basal AF-1 activity, requires the GRIP1/CBP complex or its functional equivalent.

Src potentiates CBP and GRIP1 activation functions.

The above studies suggest that Src potentiation of ER AF-1 is compatible with circumstances in which AF-1 activity is mediated by contacts with GRIP1 and CBP, but the studies are neutral as to whether Src affects the activities of GRIP1 and CBP or some other target. To gain insight into this latter question, we investigated how Src affected the transcriptional activation functions of CBP and GRIP1. GRIP1 or CBP fused to the DNA binding domain of GAL4 activate transcription when they are tethered to the GALRE reporter gene (Fig. 8C). Cotransfection of v-Src greatly increased both the transcriptional activity of CBP and GRIP1. We conclude that v-Src has the potential to change the activities of the GRIP1/CBP coactivator complex. Below we discuss the possibility that this complex may be one of the candidates for a Src/JNK target (see Discussion).

Discussion

We were encouraged to investigate whether elevated Src potentiates ER action by two observations. First, in addition to estrogen, ER activity is also stimulated by signaling cascades initiated at tyrosine kinases. Second, Src tyrosine kinase activity is often elevated in breast cancers, whose proliferation is stimulated by ER activation. Our studies indicate that expression of constitutively active forms of Src or cell stimulation with the cytokine TNFα, both of which lend to JNK activation [56,72], enhances activation of reporter gene expression by the estrogen-ER complex and powerfully enhances the otherwise weak regulation by the tamoxifen-ER complex. These effects are observed both in transiently transfected HeLa cells and in MCF-7 breast cells which express endogenous ERs. Src action upon the ER is mediated through a robust activation of the ER AF-1 function. This is seen most easily when the AF-1 region is removed from the remainder of the ER and fused to a heterologous DNA binding domain from the yeast GAL4 protein. Src action did not require the integrity of tyrosine 537 in the ER-LBD, which has been reported to be directly phosphorylated by Src [60-62]. Src tyrosine kinase activity is required for its action upon AF-1, as mutations and drugs that inactivate the kinase block the ability of ability Src to stimulate ER activity. Thus, taken together, our results suggest that elevated Src kinase activity results in elevated ER AF-1 activity.

The mechanism of Src enhancement of AF-1 activity is unusual. Previous studies have demonstrated that growth factors enhance AF-1 activity via a signal transduction pathway that is mediated by Ras, Raf, MEK, and the ERK kinases. We therefore expected that Src stimulation of ER activity might proceed through a similar pathway. However, transfection of dominant negative Ras, Raf1, and MEK mutants, or treatment of cells with PD 98059, which blocks Raf1 inputs to MEK, only partially inhibited Src potentiation of AF-1. This suggested that Src also enhanced AF-1 activity via a second pathway and, indeed, dominant negative versions of Rac, MEKK and JNKK also inhibited Src enhancement of AF-1 activity. Src cooperates both with overexpressed

ERK and overexpressed JNK to generate even higher AF-1 activity. Thus, the Src pathway leading to potentiation of AF-1 proceeds at least as much through JNKs and as it does through ERKs.

Although the pathway from Src to enhancement of AF-1 activity clearly runs through Rac/MEKK/JNKK and JNKs, it is unclear how the JNKs bring about the enhancement of AF-1 activity. One possibility is that the JNKs directly phosphorylate the AF-1 domain and thereby modify its function, as previously shown for transcription factors c-jun [73,74] and Elk-1 [75]. Our *in vitro* studies weigh against this possibility, as they indicate that JNKs fail to bind AF-1 (data not shown) and that they also fail to phosphorylate the ER A/B domain *in vitro*. Although it is possible that these failures reflect a requirement for an accessory protein not supplied *in vitro*, it is most likely an indication that the JNKs cannot efficiently phosphorylate the AF-1 domain, as activated JNKs require no accessory proteins to phosphorylate other substrates [74,76,77]. Our *in vivo* studies also suggest that JNK action is independent of direct ER phosphorylation. The major site of phosphorylation in the AF-1 region is S118 and mutation of this residue to alanine blocks AF-1 phosphorylation and enhancement of AF-1 activity by EGF, activated Ras, and other activators of ERK kinases. JNKs do not activate AF-1 via phosphorylation at S118. Replacement of S118 with alanine only partly reduces Src stimulation of AF-1 activity and the residual activation is mediated through an ERK independent pathway. We infer that this residual action is due to Src-activated JNK and this has been confirmed with overexpressed JNKs. In addition, the Src to JNK cascade targets at least two subdomains within AF-1, from amino acids 1-94, and 101-129. The first of these domains contains no potential sites for JNK phosphorylation. Furthermore, replacement of the serine cluster, including the MAP kinase phosphorylation site (S118) has no effect on the response of AF-1 to JNK activation.

How does Src-activated JNK enhance AF-1? One attractive possibility is that activated JNKs target a protein that, itself, affects AF-1 action. Recent studies indicate that AF-1 works by recruiting p160/CBP coactivators by means of a direct contact between AF-1 and the C-terminus of the p160 [24]. We have confirmed that Src enhancement of AF-1 activity can also occur in the

presence of overexpressed GRIP1 and CBP, and that this enhancement requires the C-terminus of GRIP1. Thus, one possibility is that the AF-1 mediating activities of the p160/CBP complex are the target for JNK. Accordingly, v-Src was able to enhance the transcriptional activity of both GRIP1 and CBP when they were directly tethered to DNA. One obvious question is that if, indeed, v-Src does enhance the activity of the GRIP1/CBP complex, and given that both AF-1 and AF-2 work by binding a GRIP1/CBP complex, then why would v-Src preferentially enhance AF-1 activity? We have previously shown that the AF-1 and AF-2 functions of different nuclear receptors both bind to p160s, but bind to p160 different surfaces and require different p160 transcriptional outputs [24,78]. Thus, v-Src could preferentially affect AF-1 activity by preferentially affecting a subset of p160 transcriptional inputs or outputs that are required for AF-1 action.

We stress that the sole positive evidence for Src/JNK targeting of the coactivator complex is that Src potentiates the transcriptional activation functions of both GRIP1 and CBP when they are fused to GAL4 and tethered to a promoter. We stress that this experiment is suggestive, but is only suggestive. While JNKs or something under the control of JNKs might phosphorylate GRIP1 itself, or another component of the complex (Fig. 9), there are other, equally likely, possibilities. For example, JNKs might directly or indirectly phosphorylate and modify the activities of corepressors that are suspected of modulating AF-1 action. Exploratory studies are underway to examine these and other possibilities.

Finally, we also speculate that Src enhancement of AF-1 may have consequences on cellular responses to estrogen. Breast cancer samples and cell lines almost invariably have elevated Src tyrosine kinase activity [50]. Some of those tumors and cell lines also express ER and are stimulated to grow by estrogens. In such cases, ER is believed to enhance proliferation by enhancing the expression of target genes encoding regulators of proliferation. We have shown here that overexpression of v-Src leads to increases in ER transcriptional activity in breast cells, and that this effect occurs in the presence of estradiol, the absence of hormone and, most strikingly, in the presence of tamoxifen. Thus, increases in Src kinase activity could, via

stimulation of ER transcriptional potency, enhance the ability of estrogen to induce cellular proliferation in breast cancer and breast cancer cell lines. Moreover, increased Src kinase activity could also lead to increased tamoxifen agonist activity, which might play a role in the development of tamoxifen resistance. It has been noted that tyrosine kinase inhibitors genistein and Herbimycin A inhibit cellular proliferation in response to estrogen [79-82]. Both of these inhibitors block Src activity in breast cancer cells and we also observed that these inhibitors also inhibit Src enhancement of ER AF-1 activity. It is therefore possible that both drugs could block estrogen-induced proliferation by inhibiting Src enhancement of ER action in those breast cancer cell lines and this requires investigation.

Materials and methods

Mammalian Reporter Genes and Expression Vectors

EREII-HSV-TK-CAT, a reporter gene containing two vitellogenin EREs upstream of the herpes simplex virus TR proximal promoter (-109/+45), has been previous described [83]. The GAL4 responsive reporter gene GK1 contains five GAL4 response elements upstream of a minimal adenovirus E1b promoter that has been previously described [84].

ER expression vectors have been described previously. pSG5-ER, pSG5-ERG400V [85]. pSG5-ERΔ(A/B), and Δ1-100, Δ1-116, Δ130-184, and Δ95-184 have been described previously [57]. pSG5-GAL4, -GAL4-ER(A/B), -GAL4-ER(LBD), -GAL4-VP16 expression vectors have been described [57,86]. The pSG5-ERY537R, Y537S, S118E and S118R mutants and the pSG5-GAL4-ER(A/B) mutants in which each the phosphorylation sites at Serine (S) 104, S106, S118, were mutated to Alanine (A) were created by synthesizing double-stranded oligonucleotides which encode the mutant sequence and using Quick Change Site-directed Mutagenesis Kits (Stratagene). The mutated sequences were verified by DNA sequencing using Sequenase Kits (Stratagene). The GAL-4-ER(A/B) mutant containing the triple phosphorylation site mutation (S104, 106, 118A) was made by multiple rounds of mutagenesis.

Many signal transduction molecule expression vectors were kindly provided as followings: pCMV-v-Src (Dr. M. Bishop, University of California, San Francisco); pCMV-c-SrcRF(K295R,Y527F) and pCMV-c-Src (Dr. Joan Brugge, Ariad Pharmaceuticals, Inc.); Constitutively activated pCMV-c-Src (Y527F) (Dr. Tony Hunter, Salk Institute for Biological Studies); Dominate negative pcDNA3-Rac1(S17N) and -Raf(1-257) (Dr. H. Goldberg, University of Toronto, Canada); pCMV-Flag-p38 (Dr. Roger Davis, University of Mass. Medical Center). Dominate negative Ras(S17N), MEK(K97R), pSRα-JNKK(K116R), MEKK(K432M), HA-JNK1 and HA-ERK2 have been described[56]. Coactivator expression vector pCMV-CBP was a gift from Drs. M. Rosenfeld (UCSD). pSG5-GRIP1 has been previously described [29].

Cell Culture and Transfection

HeLa cells were maintained and transfected as previously described [85]. Briefly, around 70% confluent HeLa cells were transfected with 5 μg of $(ERE)_2$-TK-CAT or 5x GAL4-Luc reporter gene, 1 μg β-galactosidase plasmid, 1 μg ER expression vector, and other coactivator and signal molecule expression vectors (2 μg) as indicated in figure legend. After 20 hrs incubation, Cells were lysed and CAT, LUC and β-galactosidase assays were performed using standard methods. The β-galactosidase activity was used to correct the variations of transfection efficiency in CAT and LUC activities. The hormones (10 nM Estradiol, ICI, Raloxifene, and 5 μM Tamoxifen) and kinase inhibitors (300 μM Genistein (Sigma), or 1 μM Herbimycin A (Sigma), and 100 μm PD 98059 (Calbiochem) were added immediately after transfection. CAT and LUC activities represented the averages from triplicate wells with less than 20% deviation. Experiments were repeated at least three times.

Western Blots

HeLa cells were transfected with ER and v-Src expression vectors, or empty control vectors, and reporter genes as described, plated and incubated overnight. Half were prepared for Western blot analysis, the remaining half was used for standard CAT and β-galactosidase assays. The following day, the transfected cells were washed in cold PBS and treated with 1ml of luciferase lysis buffer on ice for five minutes. The lysate was scraped off the dish, transferred to eppendorf tubes and pelleted in an eppendorf microfuge for 15 minutes at 4°C. Protein contents were determined and 15μg of cell proteins were separated on a 10% SDS-polyacrylamide gel and transferred to a pre-wetted Immuno-Blot PVDF membrane (Bio-Rad, Hercules CA), overnight at 90mA, 30V using a standard transfer apparatus. Following transfer, the membrane was incubated at room temperature in 5% non-fat milk in PBS-T (1xPBS, 0.1% Tween-20) for 1 hour, and washed twice in PBS-T for 10 minutes. The primary anti-ER antibody (HC-20, Santa Cruz antibodies, Santa Cruz, CA) was diluted 1:2000 in PBS-T and incubated with the membrane for 1 hour, followed by PBS-T washes, 1x15 minutes and then 2 x 5 minutes. The membrane was then incubated for 45 minutes

with horse-radish peroxidase conjugated anti-rabbit IgG (Santa Cruz antibodies) diluted 1:2000 in PBS-T, followed by PBS-T washes, 1x15 minutes and 4x5 minutes in PBS-T. After the last wash, the membrane was developed according to manufacturers instructions with a standard ECL kit (Amersham-Pharmacia Biotech), covered with Saran wrap and exposed to X-ray film.

GST-Fusion Proteins

GST-ER(A/B) and GST-cJun (amino acids 1-79, from Dr. A. DeFranco, UCSF) fusion protein were prepared as previously described [85]. Briefly, bacteria (500 ml LB media) expressing the fusion proteins were resuspended in 15 ml of TST buffer (0.5 M Tris, 1.5 M NaCl, 0.5% tween 20, pH=7.5) and sonicated mildly for 2-3 min. in ice. The debris was pelleted at 12,000 rpm for 1 h in an ss34 rotor. The supernatant was rotated gently for 2 hrs at cold room with 0.5 ml of glutathione sepharose 4B beads that had been prewashed with 5-10 vol. of TST buffer. GST-fusion proteins beads were washed with 10-20 vol. PBS 0.01% Nonidet P-40 and resuspended in 1:1 vol. of IPAB-150 (20 mM HEPES, 150 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitors, pH 7.9) for storage at 4 °C until use.

In Vitro Kinase Assay

Src, MAP, and JNK kinase assays were carried out following the manufactory's protocol (Upstate Biotechnology) with purified Src, MAP, JNK kinases, as provided. Briefly, purified Src, MAP, JNK kinases were mixed with the corresponding substrates or GST-ER(A/B), the reaction started by adding the corresponding reaction buffer containing (γ-^{32}P)ATP, then mixed gently and incubated at 30 °C for 15-30 min. After addition of 40% TCA or 2x SDS-PAGE loading buffer to stop the reaction, the phosphorylated kinase substrates and GST-ER(A/B) were detected by liquid scintillation counter or autoradiography of 10-12% SDS-PAGE.

Acknowledgments

We thank David Stokoe for helpful comments on the manuscript. This work was supported by Public Health Service grants DK51083 and CA 80210 (to PJK) and by grants from the California Breast Cancer Research Program (to PJK and MK).

Peter Kushner and John Baxter wish to inform readers that they have significant financial holdings in and are consultants to KaroBio AB, a pharmaceutical company with interests in nuclear receptors.

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Figure Legends

Fig. 1. v-Src potentiates estrogen receptor AF-1 transcriptional activity. **(A)** Activity of an ERE -CAT reporter gene in HeLa cells transfected with expression vectors for full length ER (G400V derivative), and for v-Src as indicated in the presence of ligands, estradiol (E_2), tamoxifen (Tam), ICI 182,780 (ICI), and raloxifene (Ral). Inset shows basal activities on an expanded scale. **(B)** Western blots were performed upon extracts of cells that were transfected with different amounts of ER expression vector, or control vector, +/- transfected v-Src. The lower panel shows transcriptional activation of the ERE responsive reporter gene as a function of ER levels +/- transfected v-Src. **(C)** Activity of the ERE reporter gene in HeLa cells transfected with an expression vector for ER, the indicated amount of expression vector for v-Src after treatment with tamoxifen (closed circles) or vehicle (open boxes). Fold inductions were determined by comparing the relative CAT activity in the presence of v-Src to that in the absence of v-Src (set as 1 fold). **(D)** Activity of the ERE-CAT reporter gene in MCF-7 breast tumor cells which express endogenous ERs. **(E)** Activity of a reporter gene with five GAL4 response elements in cells transfected with expression vectors for GAL4 fusion proteins to the ER A/B region (GAL4-AB), to the ER ligand binding domain (GAL-LBD), or to the herpes virus VP16 activation domain (GAL4-VP16) in the presence or absence of co-transfected v-Src. Fold inductions were determined as in Fig. 1C.

Fig. 2. Src action upon ER is independent of tyrosine 537. Activity of the ERE-CAT reporter was determined in the presence of empty pSG5 expression vector, or expression vectors for ER, ERG400V, ERY537R and ERY537S, both in the presence of an empty expression vector or an expression vector for v-Src.

Fig. 3. Src action on ER AF-1 requires Src tyrosine kinase function. Shown is fold induction of the GALRE reporter gene activated by GAL-ER(A/B) in the absence of Src (normalized to 1) and by c-Src, by constitutively active c-Src (c-Src (Y527F)), by an inactive derivative of c-Src (c-Src(K295R, Y527F)),

or by v-Src in the absence or presence of tyrosine kinase inhibitors (Genistein or Herbimycin). The effect of the inhibitors on v-Src-enhanced and basal stimulation by GAL-Elk1is shown in the inset..

Fig. 4. v-Src activates AF-1 through ERK and JNK MAP kinases. **(A)** The ER A/B domain is a target for ERK but not Src kinase. Assay of kinase activity using purified Src and ERK kinases, a peptide Src substrate (amino acids 6-20 of cdc-2), PHAS-I (a peptide ERK substrate), and purified GST-ER (A/B) fusion protein , as indicated. **(B)** v-Src action on AF-1 is partly blocked by dominant negative Ras (Ras(S17N)), Raf1 (Raf(1-257)), MEK (MEK(K97R)), or the PD 98059 compound that blocks activation of ERK MAP kinases. Induction of a GALRE reporter gene activated with GAL-ER (A/B) and v-Src in the presence of the indicated dominant negative or drug inhibitor. Inset on right shows the expected complete blockade of EGF action on Elk-1 by the dominant negatives and PD compound. **(C)** v-Src action on AF-1 is also blocked by dominant negative Rac (Rac(S17N)) or MEKK (MEKK(K432M)). An assay similar to B of reporter gene response to Src in the presence of the indicated dominant negative inhibitor. **(D)** TNFα and Src action on AF-1 is blocked by dominant negative JNKK(K116R). Induction of the GALRE reporter gene activated by GAL-ER(A/B) in HeLa cells co-transfected with dominate negative JNKK (JNKK(K116R)) as indicated. After 20 hours incubation, the cells were treated with TNFα for 1 hour or transfected with v-Src expression vector, as indicated, then incubated for 6 hrs before assay. **(E)** ERK2 and JNK1, but not p38, cooperate with Src to activate AF-1. Induction of the GALRE reporter gene activated by GAL-ER(A/B) in the presence of activated Src and elevated ERK2, JNK1, or p38 as indicated.

Fig. 5. Src activated ERKs, but not JNKs, require S118 in the A/B domain to activate AF- 1. **(A)** Src potentiation of AF-1 is in part independent of S118. Induction of the ERE responsive reporter gene activated by ER, or ERS118E and ERS118R, with and without transfected v-Src. **(B)** Induction of the GALRE reporter gene activated by GAL-ER(A/B) or the indicated mutant thereof, with v-Src. v-Src induction of wild type GAL-ER(A/B) was set as 100%. **(C)** Src activation of AF-1 that is independent of S118 is resistant to PD 98059, the ERK pathway inhibitor. Src effects are shown as fold induction of

reporter gene activity. **(D)** Src potentiation on ER(A/B) with mutations in S118 serine cluster cooperates with JNK but not ERK kinases. Induction of the GALRE reporter gene activated with GAL-ER(A/B) S118, v-Src, and vectors for JNK1 or ERK2, as indicated.

Fig. 6. Src potentiates transcription from two separable subdomains of AF-1. **(A)** Fold induction that v-Src elicits from the ERE reporter gene activated by the indicated ER derivative in the presence of tamoxifen. **(B)** The sensitivity of v-Src enhancement of tamoxifen response to PD 98059 was determined in the presence of the indicated ER derivative in the presence of tamoxifen. All values (after subtraction of backgrounds) were normalized to that obtained in the presence of fully wild type ER, which was set at 100%.

Fig. 7. ER(A/B) is not phosphorylated by JNK1 *in vitro*. GST-cJun or GST ER(A/B) was *in vitro* phosphorylated by immunopurified JNK1 kinase extracted from HeLa cells treated with TNFα (a Src/JNK activator), and analyzed on 10% SDS-PAGE with autoradiography. Control experiments (see, Fig. 3) indicate that GST-ER(A/B) is a good substrate for ERKs.

Fig. 8. Src/JNK potentiates ER AF-1 mediated by GRIP1 and CBP. **(A)** Elevated GRIP1 or CBP cooperate with Src in activation of AF-1. Left panel shows induction of the GALRE reporter gene activated with ER-GAL(A/B) and the effects of transfected v-Src GRIP1 or CBP as indicated. Right panel shows the substantial effects of GRIP1 on v-Src potentiation of the triple serine ER-AF-1 mutant even in the presence of PD 98059 as indicated. **(B)** A mutant of GRIP1 deficient for mediating basal AF-1 acts as a partial dominant negative for Src action on AF-1. Response of the reporter gene to v-Src in the presence of elevated amounts of the indicated derivative of GRIP1. GRIP1(1-1121) is missing amino acids 1122-1462 and does not bind to ER(A/B) or mediate AF-1 action in the absence of Src. **(C)** CBP and GRIP1 activation functions are potentiated by Src. Activity of the GALRE:luciferase reporter gene activated by GAL4 fusions to CBP or GRIP1 as indicated and cotransfected with expression vector for v-Src.

Fig. 9. Pathways of v-Src stimulation of ER(A/B) transcriptional activity. One pathway leads from Src through Ras, Raf, MEK, and ERKs and results in phosphorylation of the ER (A/B) on S118. The second pathway leads to Rac, MEKK, JNKK, and JNKs. JNKs do not phosphorylate S118. Among many possibilities JNKs may target coactivators that mediate AF-1 activity. This is indicated by an arrow from JNK to Coactivators. That it is merely a possibility is indicated by the question mark.

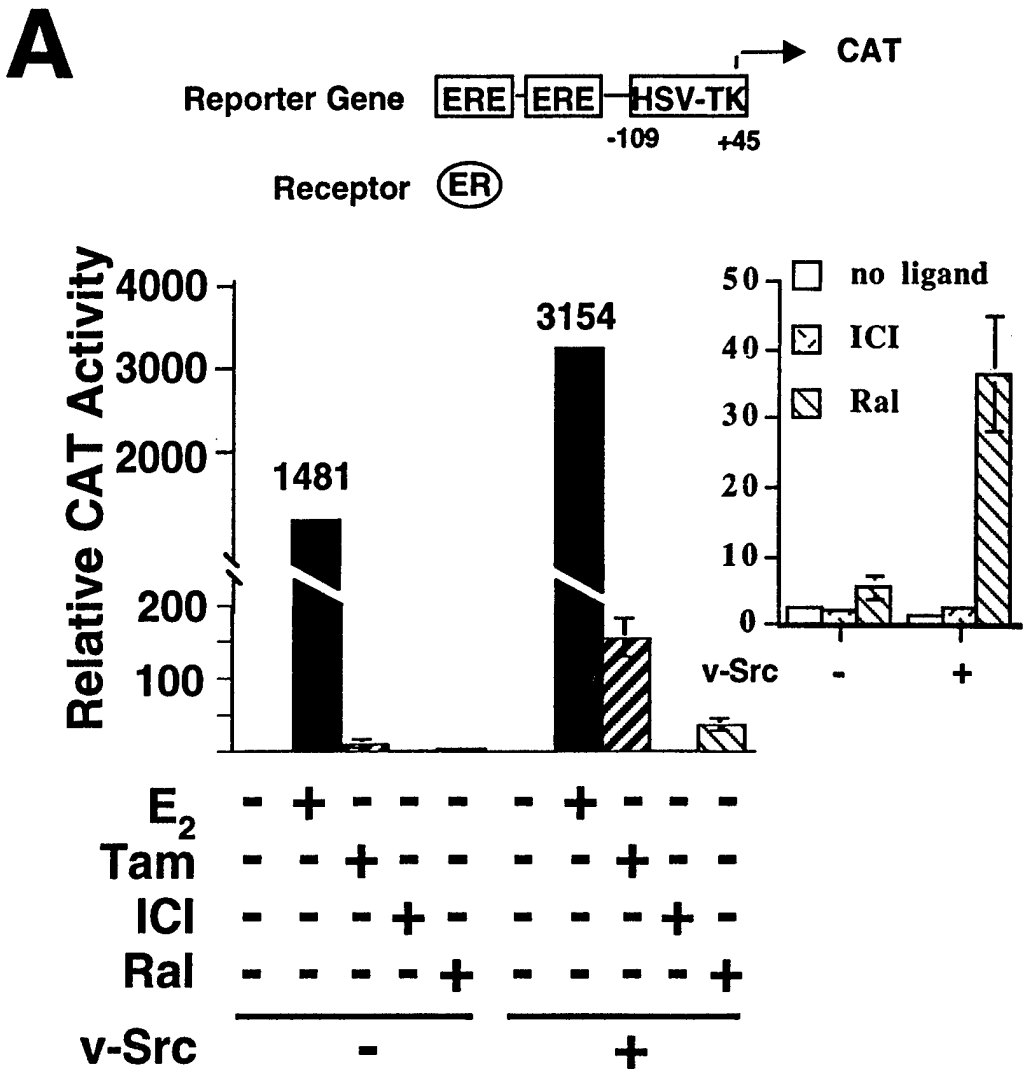


Fig.1A, W.Feng, et al.

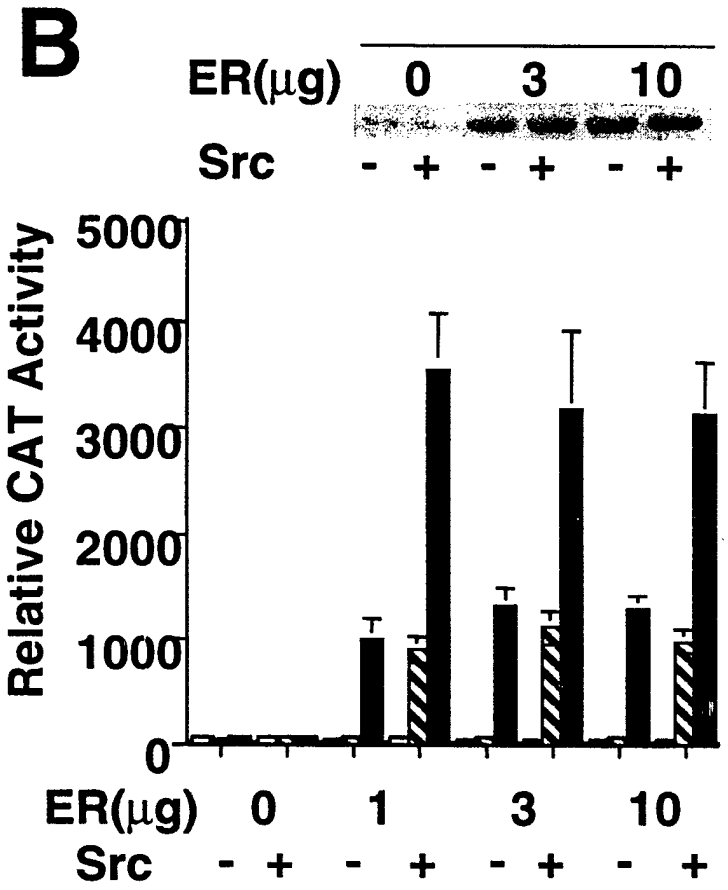
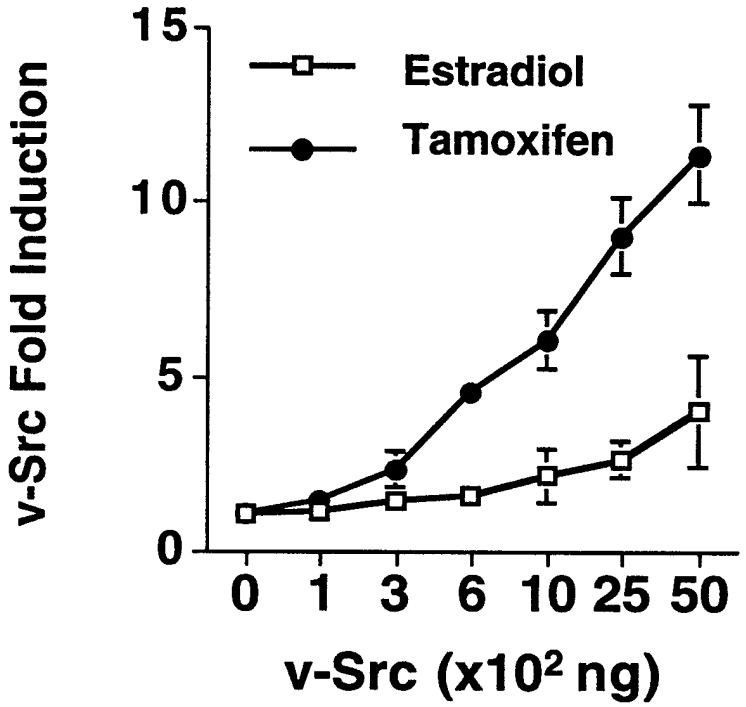


Fig. 1B, W. Feng et al

C



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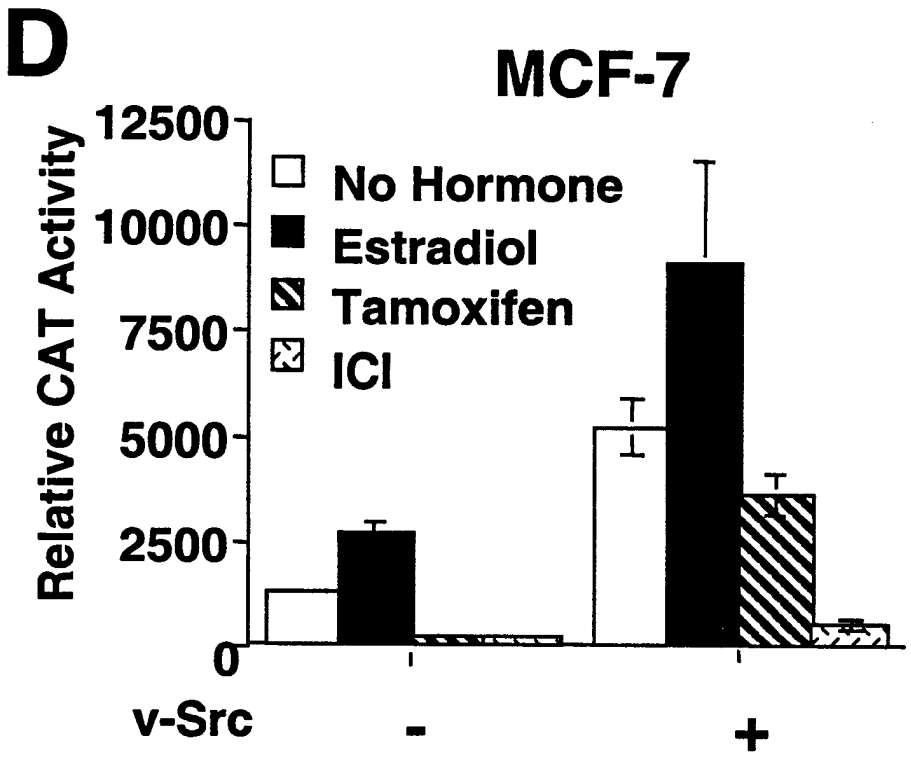

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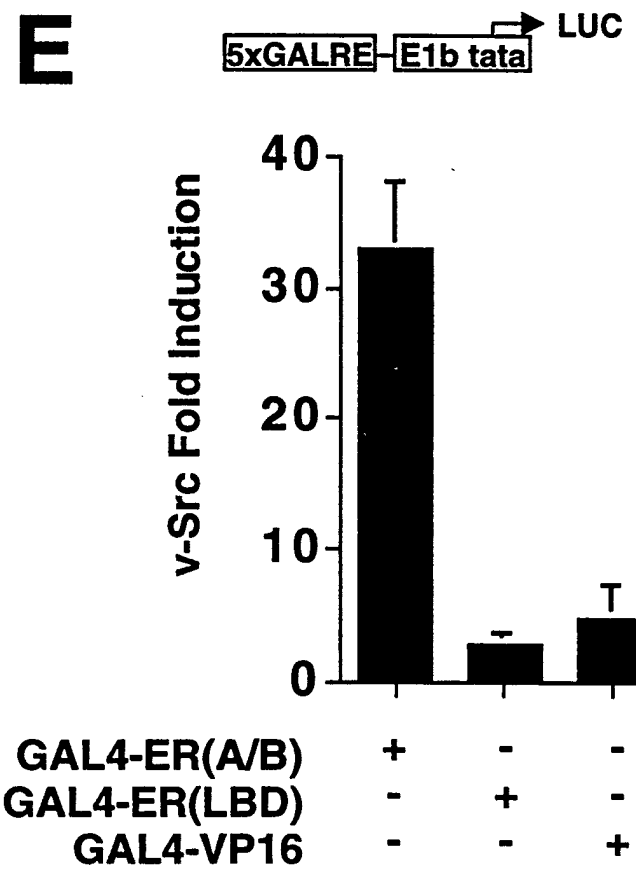

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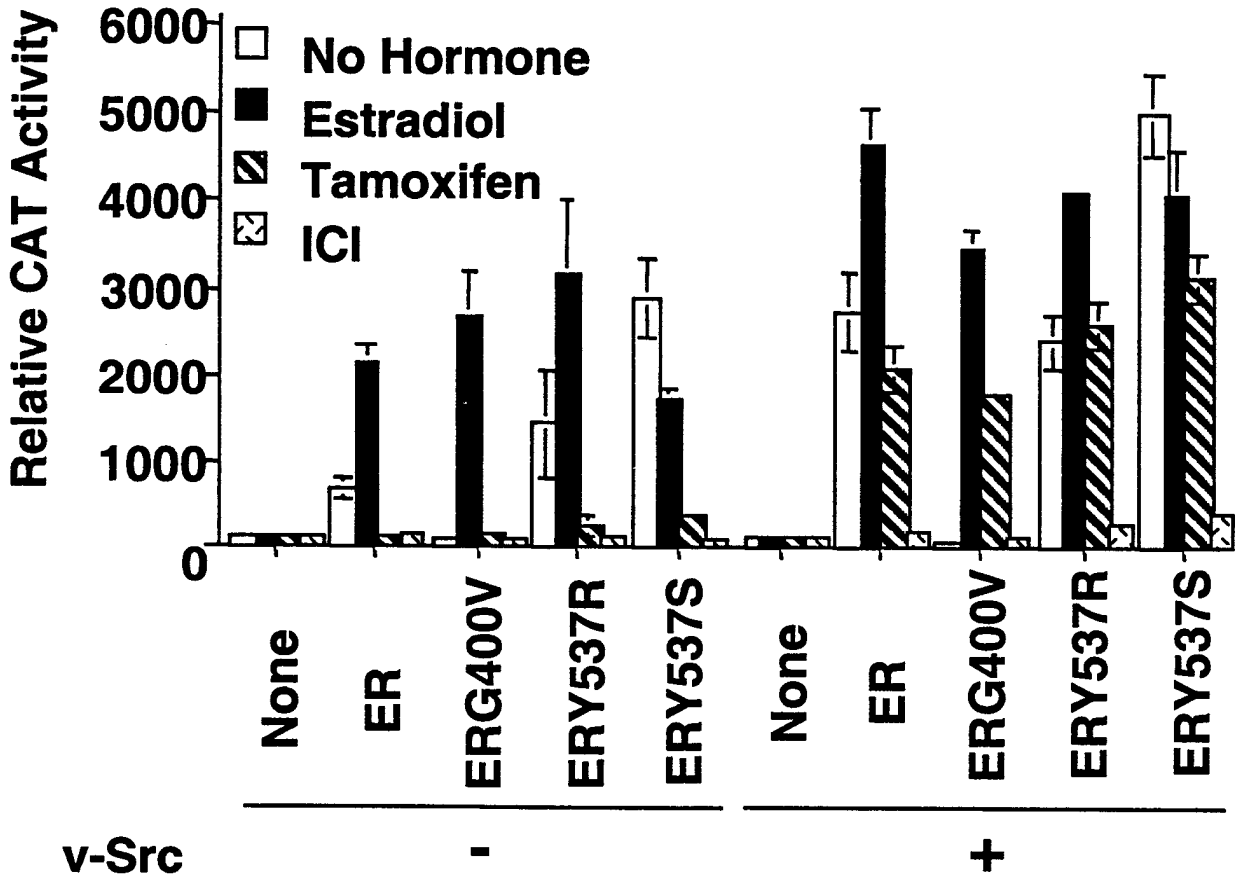
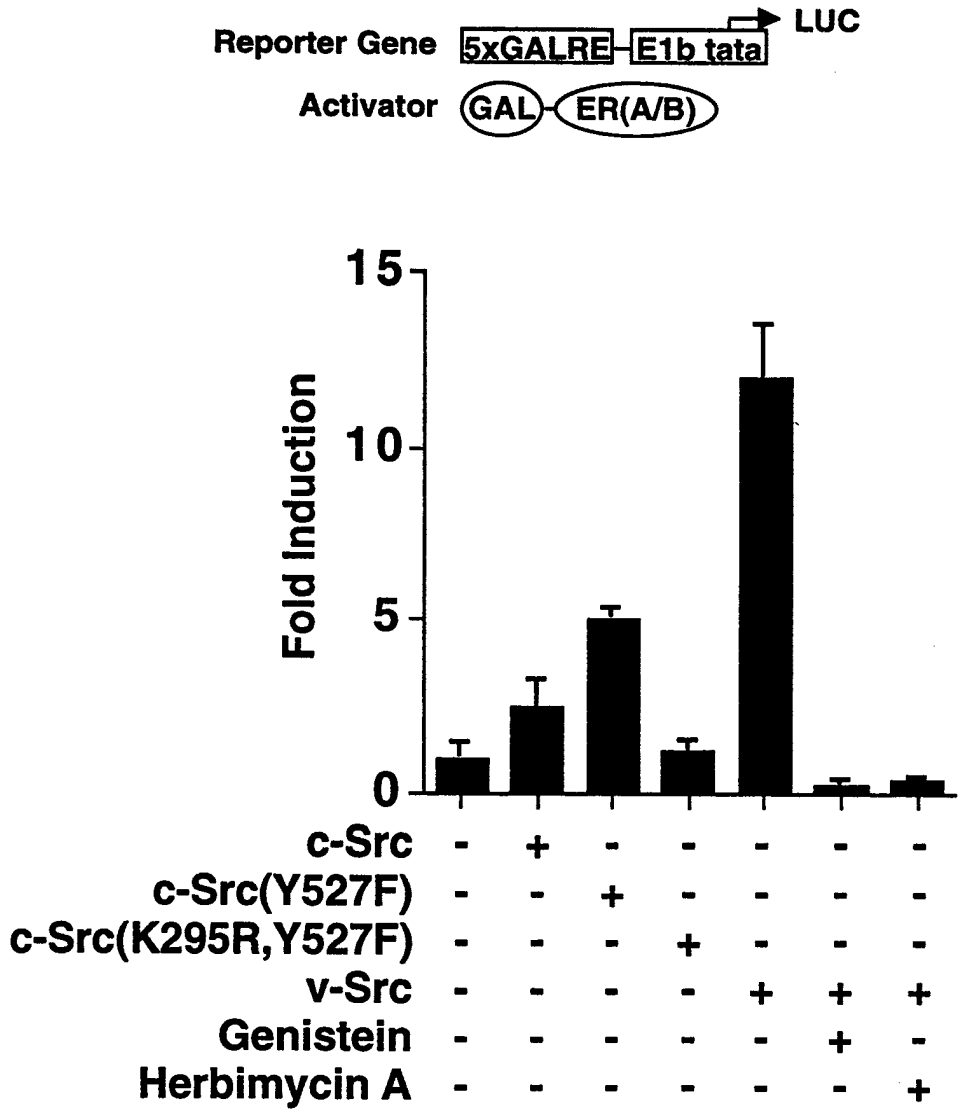


Fig. 2 W. Feng, et al.



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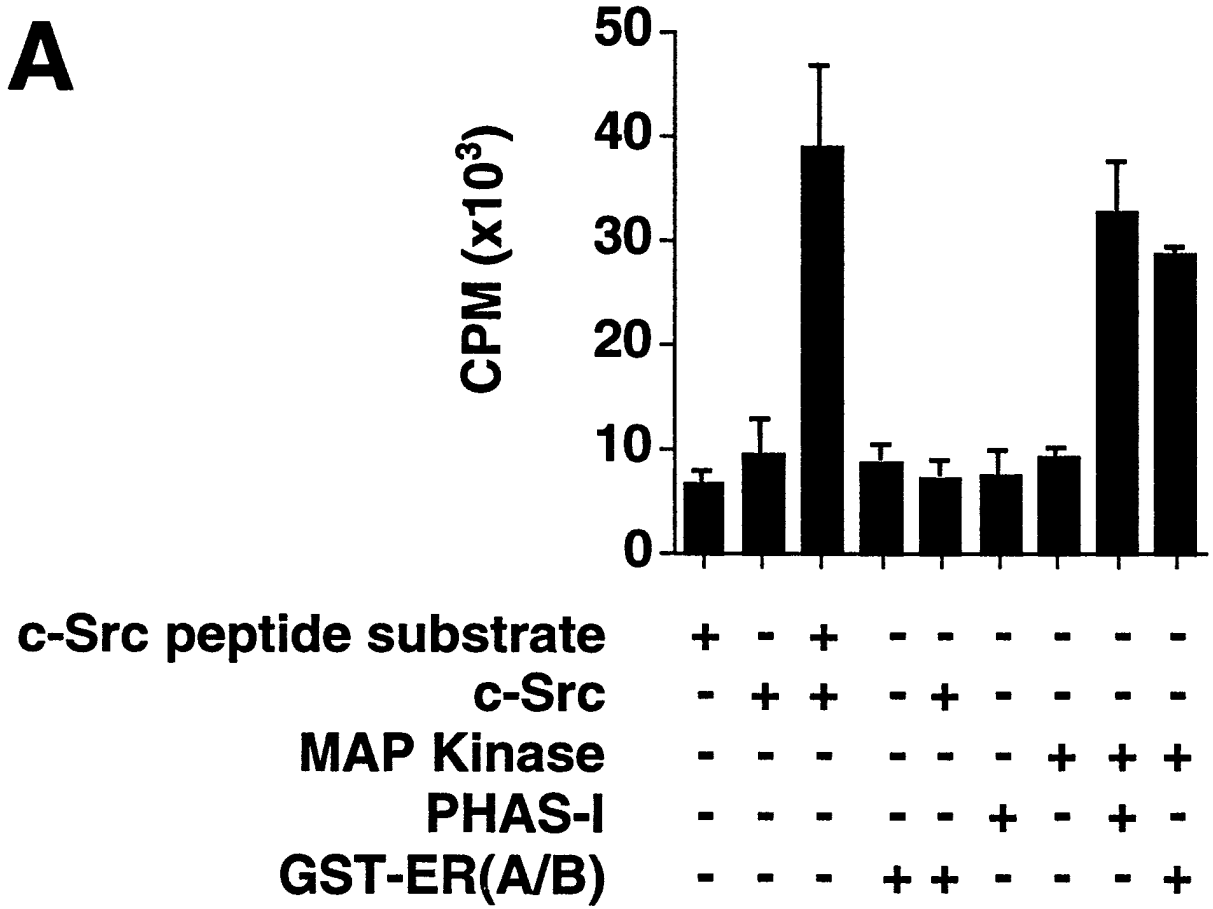

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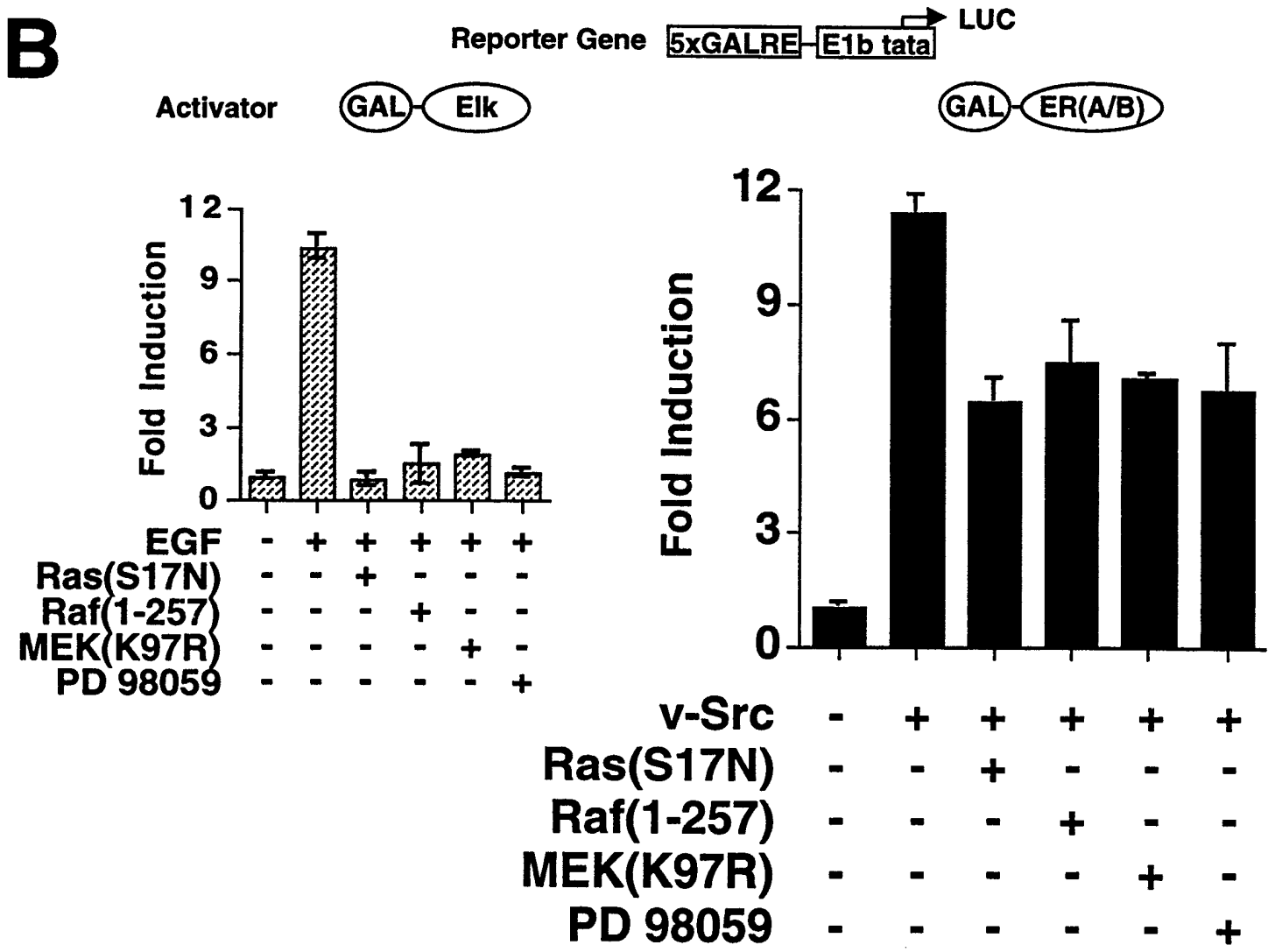

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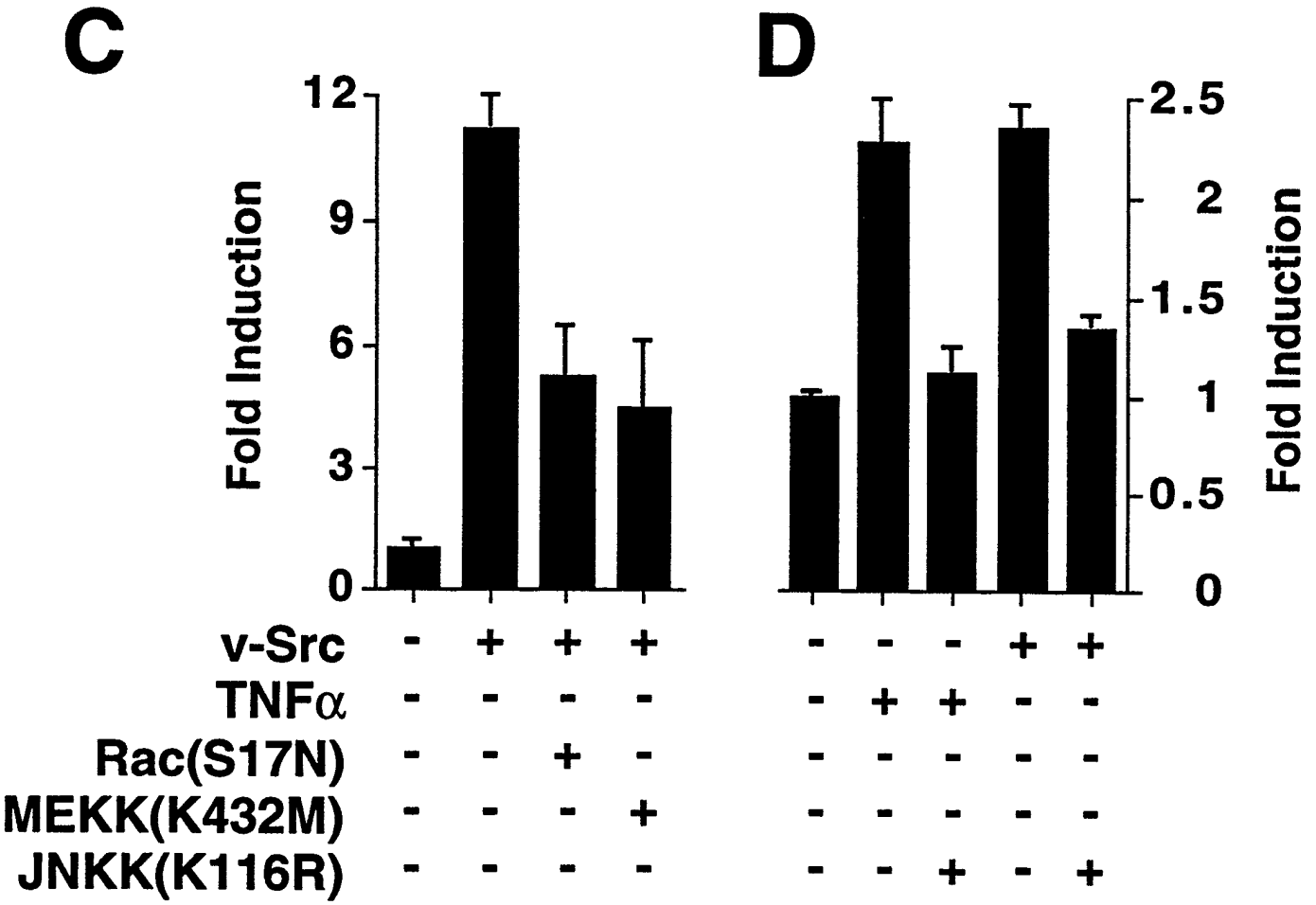

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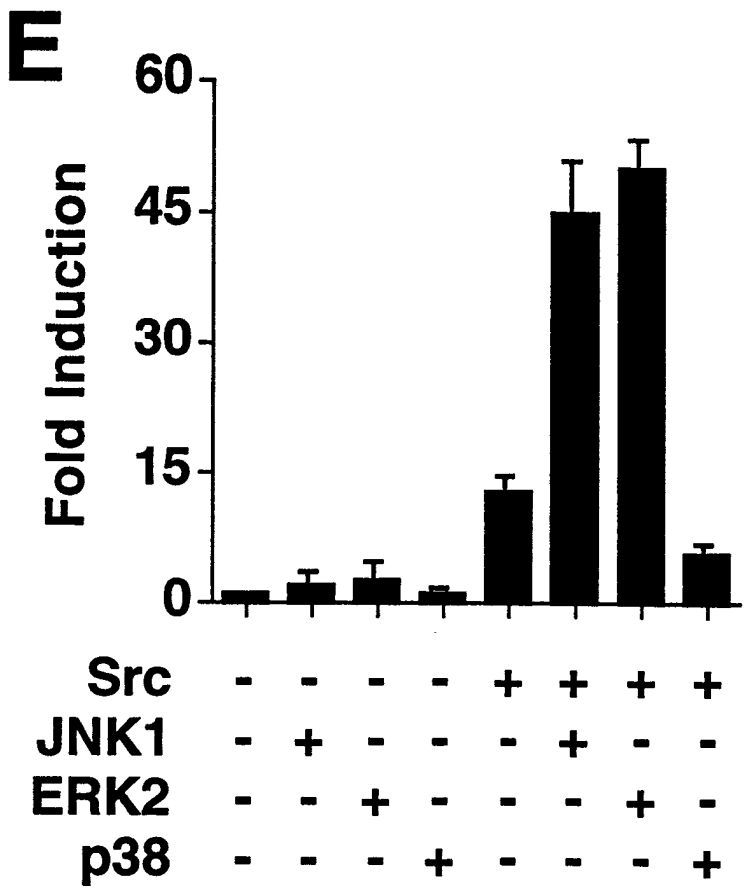

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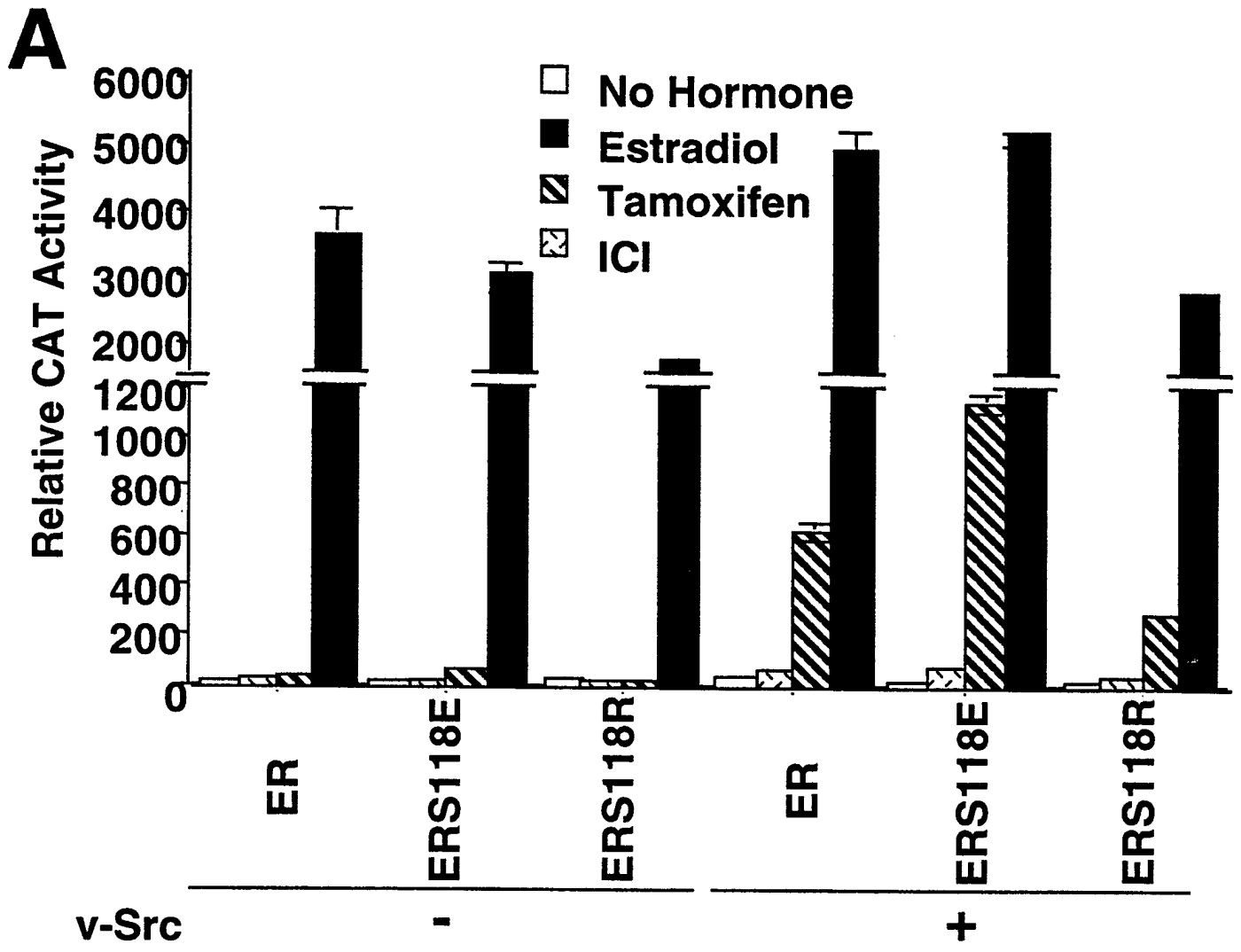
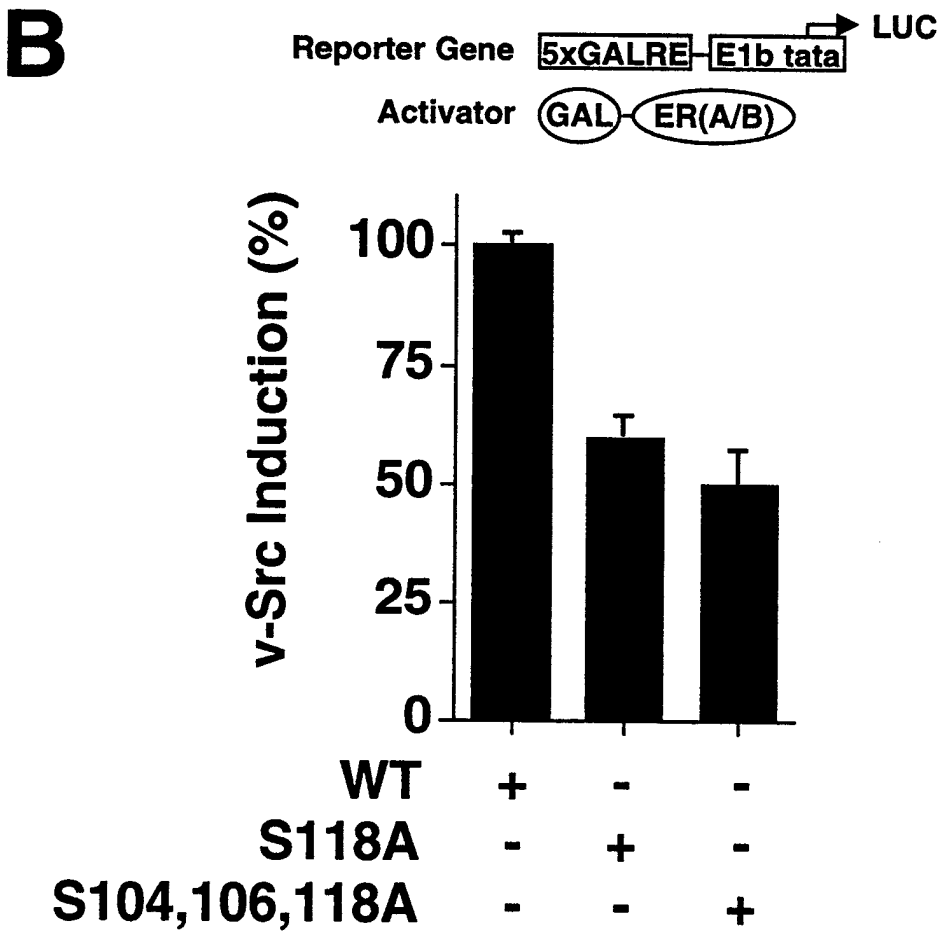


Fig. 5A W. Feng, et al.



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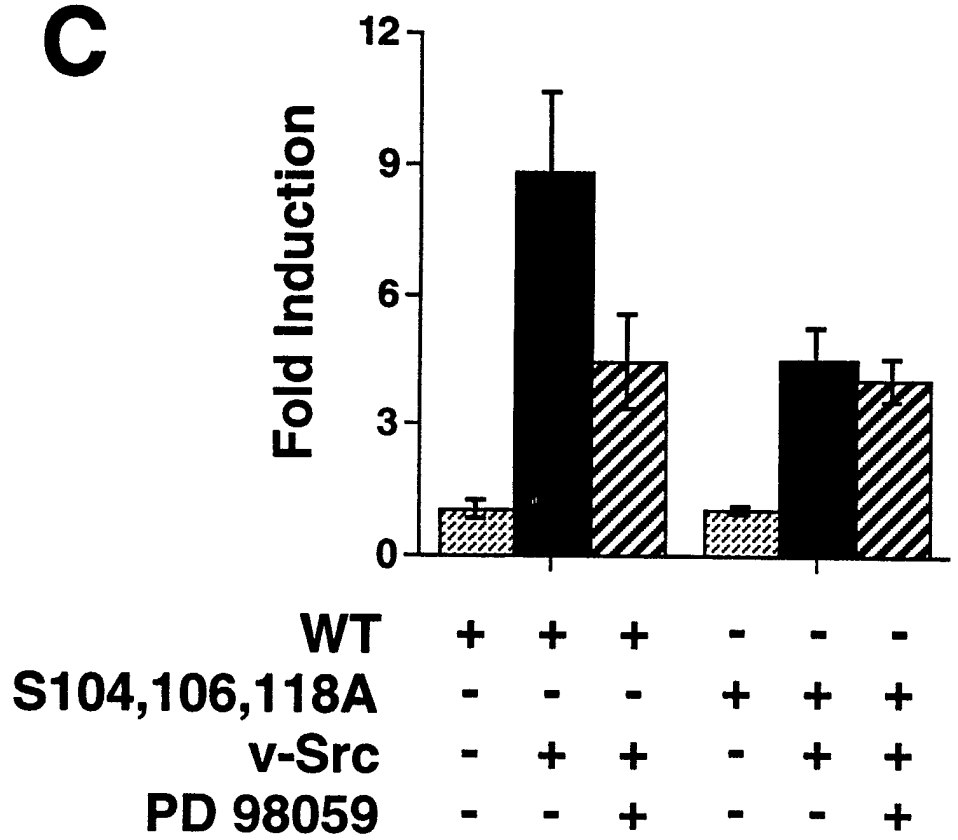

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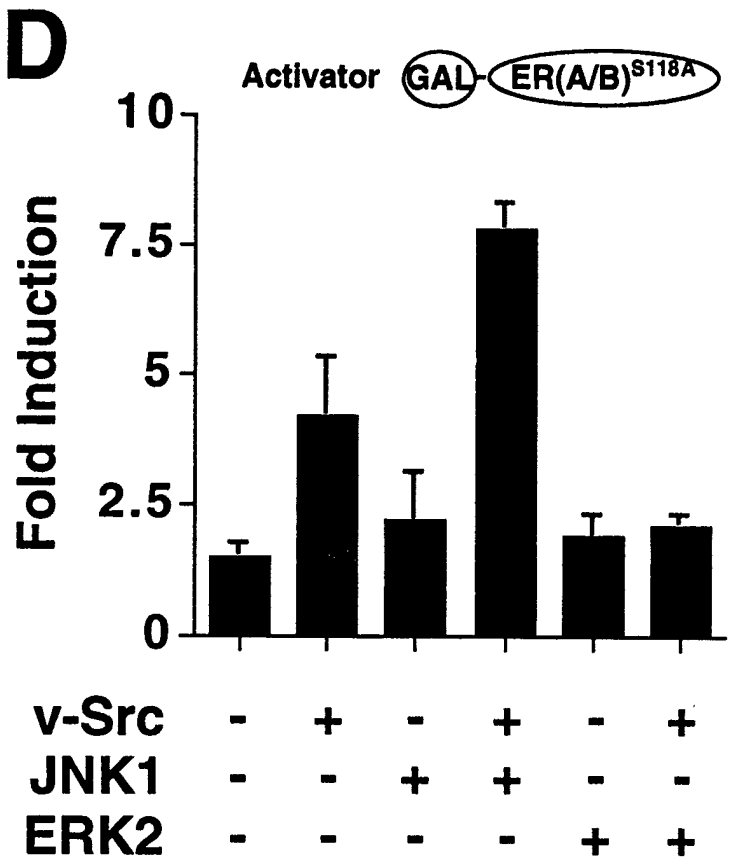

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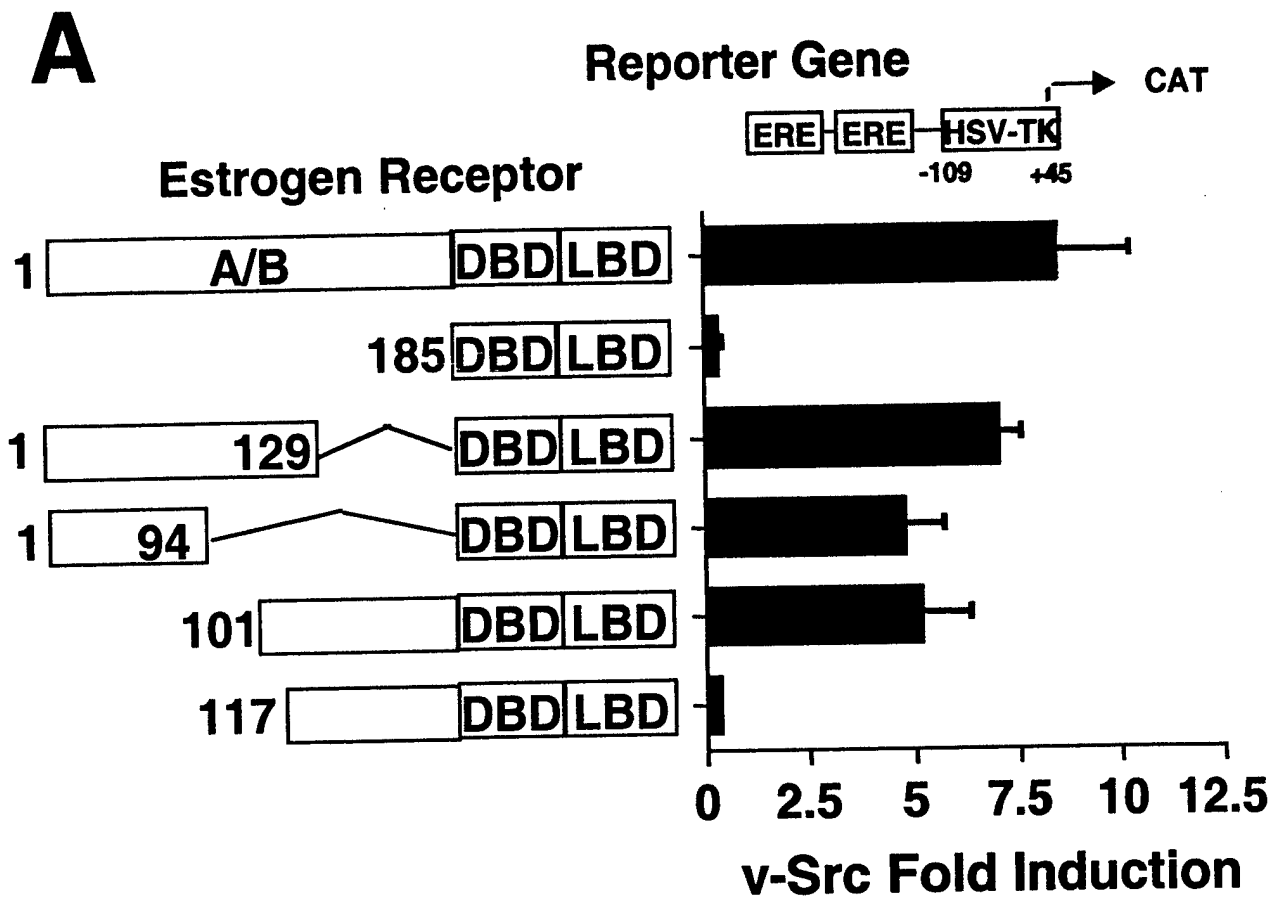

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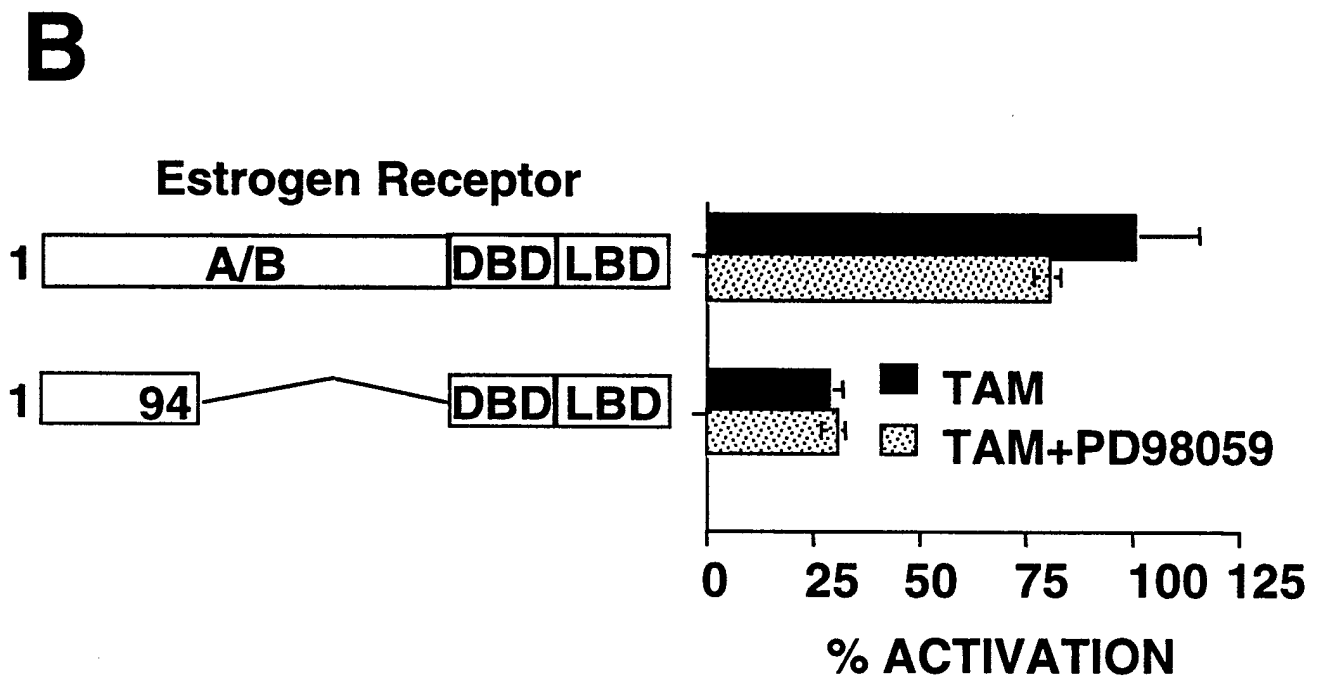

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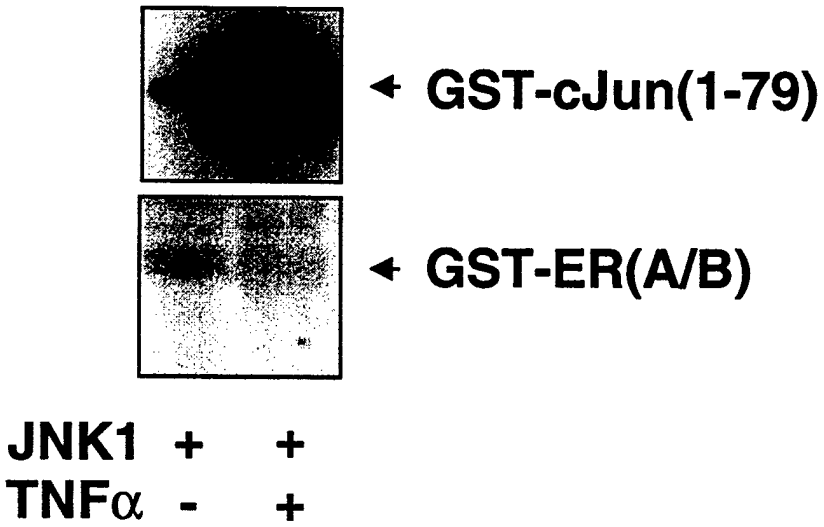

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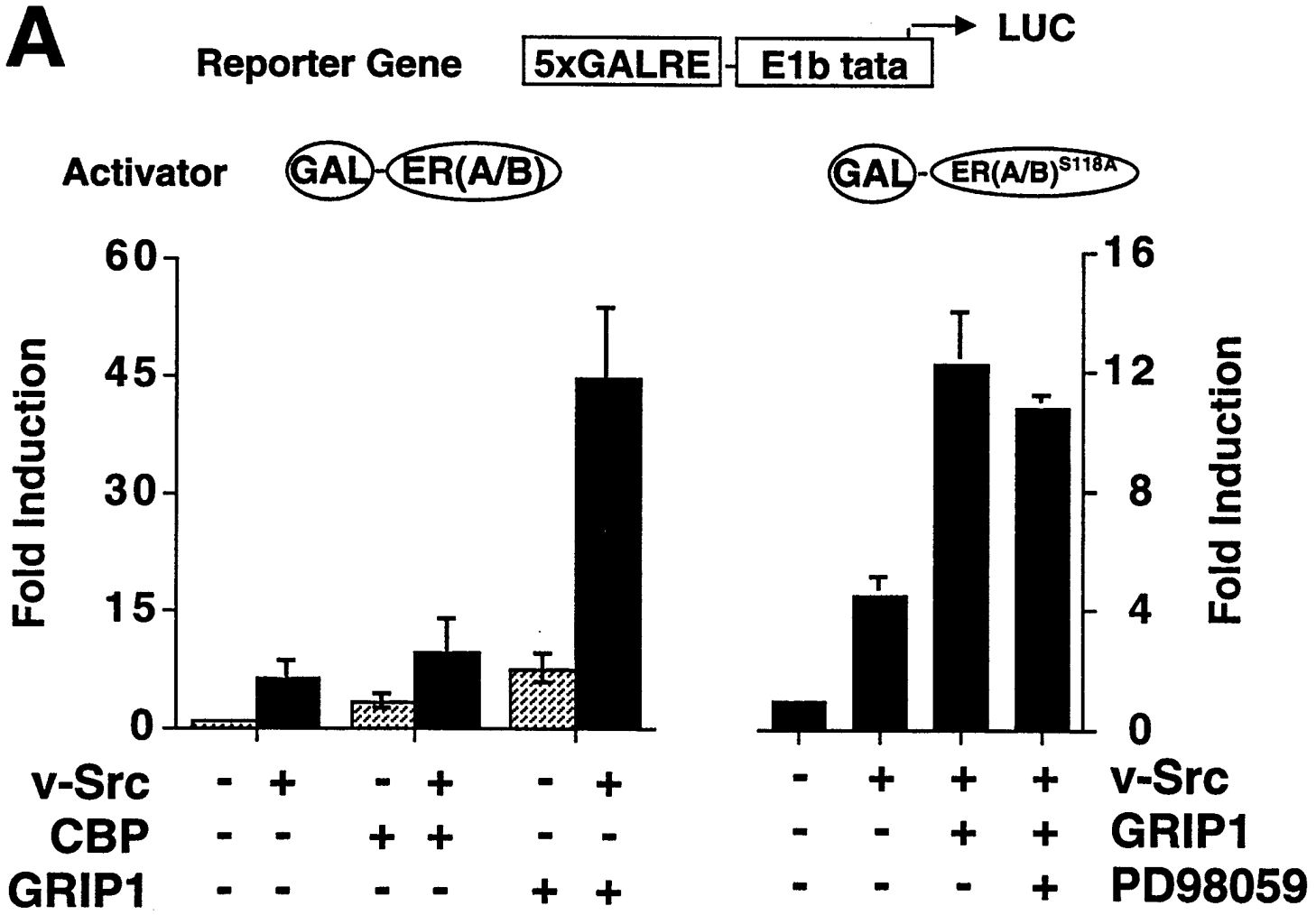

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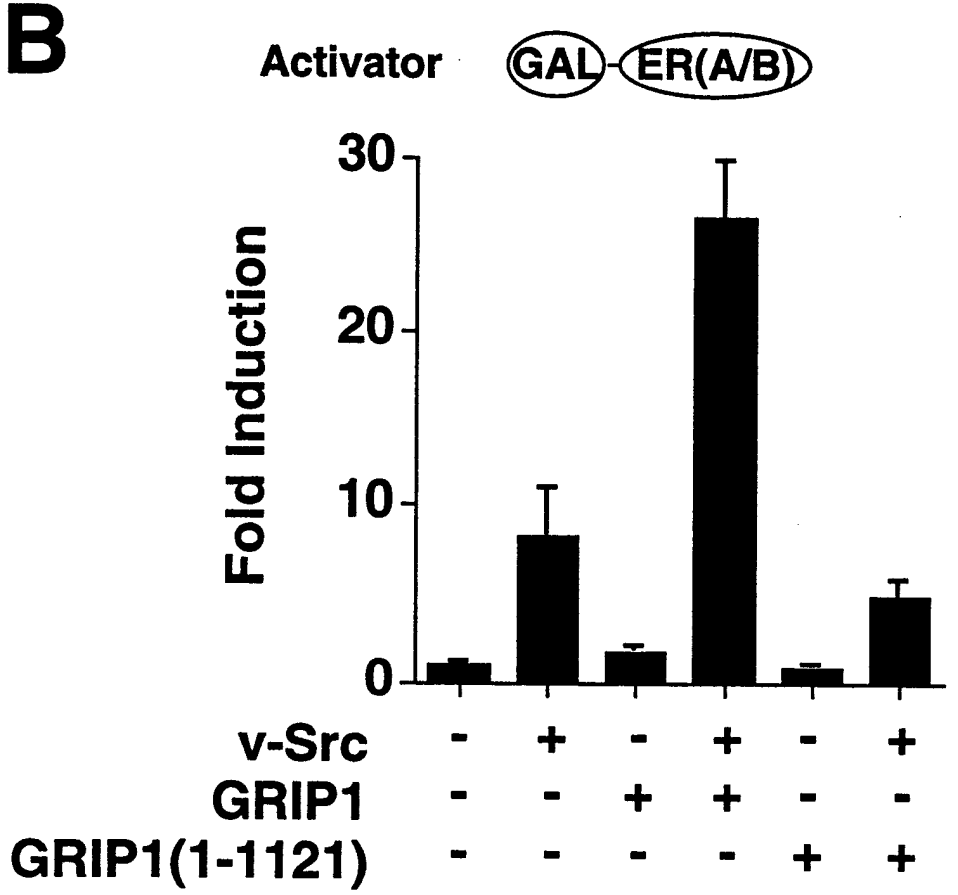

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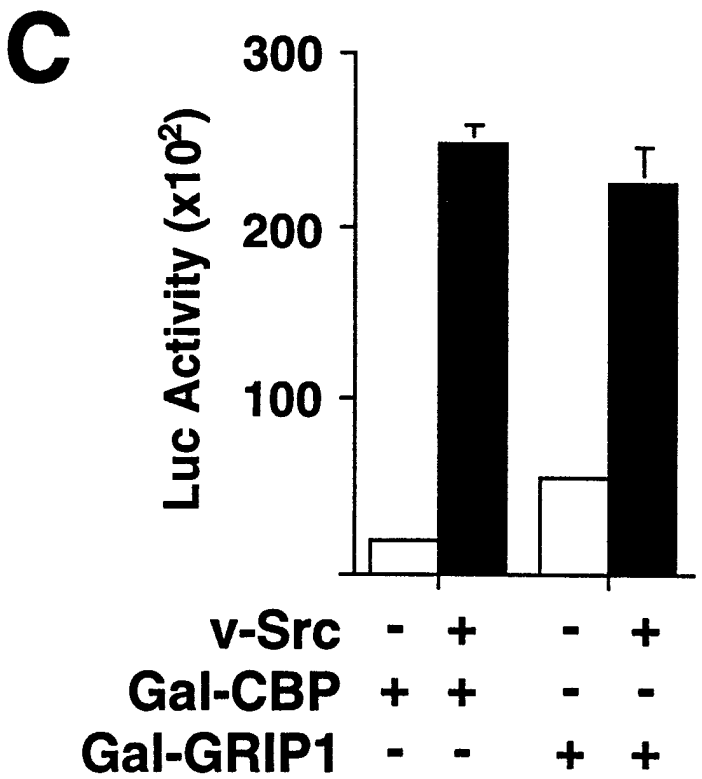

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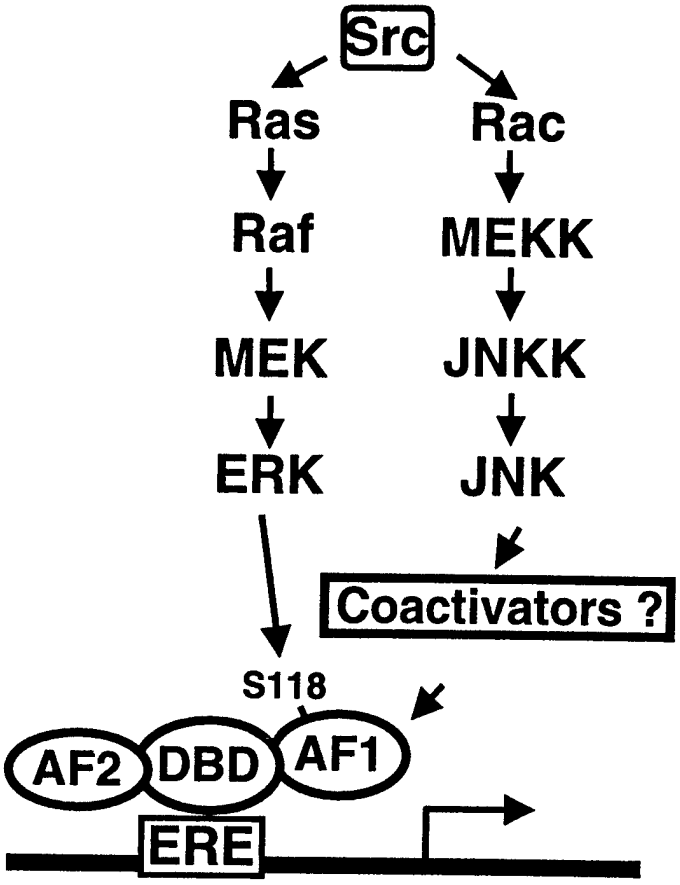

Fig. 9. W. Feng et al.